Differences in Inflammatory Responses to Exposures of Concentrated Ambient Particles in Susceptible Volunteers

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ABSTRACT

Respirable particulate matter (PM) is associated with harmful cardiopulmonary effects in humans. To test the hypothesis that individuals with certain susceptibility factors have heightened inflammatory and airway responses to PM exposure, a single-blind randomized dose crossover human study of controlled exposure to filtered air (FA), and concentrated ambient particles (CAPS) was conducted. We enrolled 10 mild- moderate asthmatic GSTM1 null subjects, 10 mild- moderate asthmatic GSTM1 present subjects and 10 healthy GSTM1 present subjects to determine the short-term effects of CAPS exposure in individuals likely to be at risk for adverse effects. Outcome measures included symptom scores, physiologic measures (vital signs, spirometry, exhaled nitric oxide, heart rate variability) as well as serum, sputum, and nasal lavage samples for inflammatory biomarkers. Particle mass concentrations averaged 187$\mu g/m^3$ for CAPS and 35 $\mu g/m^3$ for FA during the 2-hour exposures. During both CAPS and FA exposures, GSTM1-null asthmatics reported increased symptom scores while decreased systolic blood pressure was observed in all groups. Mean exhaled nitric oxide concentration (FeNO) was increased immediately after CAPS exposure compared to FA for all subjects. Sputum total cell counts trended higher after CAPS than after FA exposures and nasal lavage IgG4 was increased after CAPS and decreased after FA exposure for the entire population. Heart rate variability (HRV) data demonstrated increased heart rate and decreased HRV post-exposure across all groups regardless of exposure conditions (CAPS or FA). CAPS exposure and susceptibility group showed minimal effects on HRV changes. Overall, a few endpoints supported the hypothesis of increased airway inflammation with CAPS exposure. However, the results did not demonstrate an effect of asthma or GSTM1 status on the inflammatory response to CAPS.

EXECUTIVE SUMMARY

Background

A large body of scientific evidence has established that particulate air pollution has detrimental cardiopulmonary effects on human health. Multiple studies have identified particles from motor vehicle combustion engines as having a particularly important impact on human health. Accordingly, regulatory and monitoring policies have been established to limit particulate matter (PM) levels in the ambient air. These air quality standards have been based on yearly or daily averages. However, previous studies have illustrated that short-term exposure to some types of PM can lead to adverse health effects. Thus, there is a need for increased understanding of how ambient PM may impact human health over short exposure time periods. Animal, cellular and human epidemiological studies all confirm that short-term exposure to PM can cause airway inflammation. However, human studies that have directly tested short-term PM exposure on human subjects have reported only modest, subtle changes in disease markers likely because of the great heterogeneity in responses observed. A few studies show that marked significant changes do occur but only in some subjects. Thus, it becomes important to study individuals with risk factors for susceptibility or responsiveness to PM. Based on available data, two prominent "susceptibility factors" (GSTM1 null genotype and asthma) have been selected for study. These susceptibility factors, specific genotype (presence of the *GSTM1* null polymorphism) and underlying pulmonary disease (asthma) are very common (50% and 8% respectively). By investigating these “at-risk” populations specifically, it is possible to determine the impact of PM on a large segment of the population which is potentially most vulnerable to the adverse health effects of air pollution. Such studies may allow future air quality regulations to not only protect the health of the general public, but to additionally protect the health of subpopulations at significantly greater risk. Additionally, this study will address a knowledge gap regarding the inflammatory effects of fine concentrated ambient particle (CAPS) exposure in susceptible subpopulations as previous similar work has predominantly focused on diesel exhaust particles (DEP). **The primary objective of this study is to test the hypothesis that individuals with certain ‘susceptibility factors’ will have heightened inflammatory and airway responses with exposure to concentrated ambient particles (CAPS).**

Methods

We conducted a single-blind randomized crossover study of controlled exposure to filtered air (FA) and to concentrated ambient fine particles (CAPS), in 10 GSTM1 null mild-moderate asthmatics, 10 GSTM1 positive mild-moderate asthmatics, and 10 GSTM1 positive healthy subjects. All human subject procedures were approved by the appropriate institutional review boards at Los Amigos Research & Education Institute (LAREI) and University of California, Los Angeles (UCLA). Each subject completed the study protocol with a total of 5 visits: 1 screening visit, 2 exposure days, and 2 follow up visits 1-day post-exposure. Experimental exposures were separated by at least 2 weeks. Each subject was exposed in a whole-body chamber to CAPS (PM2.5) at a target concentration of 200 $\mu g/m^3$ monitored in real time by a nephelometer and controlled by diluting the output of the ambient fine particle concentrator with varying amounts of filtered air. Exposures lasted two hours, with submaximal exercise (approximately tripling resting ventilation) for 15 min of every half-hour. Outcome measures included symptom scores,

physiologic measures (vital signs, spirometry, exhaled nitric oxide, heart rate variability) as well as serum, sputum, and nasal lavage samples for inflammatory biomarkers.

Results

Thirty-one subjects enrolled in the study and thirty subjects (10 GSTM1-null asthma subjects, 10 GSTM1-present asthma subjects, 10 GSTM1-present healthy subjects) completed the protocol. No serious adverse events occurred. Particle mass concentrations averaged 187$\mu g/m^3$ for CAPS and 35 $\mu g/m^3$for FA during the 2-hour exposures. Overall, few significant CAPS-attributable changes were observed for physiologic and symptom endpoints, consistent with findings in previous studies using similar exposures. An unequivocally significant relative increase in FeNO was associated with CAPS exposure for all groups, without significant changes in most concurrent respiratory or systemic inflammatory markers. Sputum total cell counts trended higher after CAPS than after FA exposures and nasal lavage IgG4 was increased after CAPS and decreased after FA exposure for the entire population. GSTM1-null asthmatics reported increased symptom scores during both CAPS and FA exposures. Post –exposure systolic blood pressure decreases were observed in all groups for both FA and CAPS exposures. Heart rate variability (HRV) data showed increased heart rate and decreased HRV post-exposure across all groups regardless of exposure conditions (CAPS or FA). CAPS exposure and susceptibility group showed minimal effects on HRV changes. Overall, some data supported the hypothesis of airway inflammatory responses to CAPS exposure, but these responses were not significantly different between subject groups. Thus, within the limitations of the study design, the data does not support the hypothesis that individuals with mild-moderate asthma or GSTM1-null genotype have increased susceptibility to the inflammatory effects of short-term CAPS exposure.

Conclusions

In summary, the study findings do not support the hypothesis that human subjects with mild-moderate asthma or GSTM1-null genotype have greater inflammatory responses to short-term CAPS exposure at levels approximating 200$\mu g/m^3$ for 2 hours. If such responses are influenced by asthma status and GSTM1 genotype, the influences appear to be subtle and were not detected by the instituted study design. Identification and characterization of subpopulations susceptible to the adverse health effects of particulate air pollution remains a critically important area of research. Based on our findings, future exposure study designs should consider factors of increased power from larger subject enrollment, potential alternatives to spirometric changes as primary study endpoints, increased CAPS exposure (higher concentration and/or greater duration), cautious and ethical inclusion of more clinically severe asthmatics in exposure studies, and consideration of additional genetic and host co-factors such as diet that may modulate inflammatory response to oxidative stress. Additionally, our current study data shows relative increases in FeNO with CAPS exposure suggesting potential utility of this measurement as an early sensitive marker of airway inflammatory responses to fine particle exposure in both healthy and asthmatic individuals. Inclusion of FeNO measurement in future fine CAPS exposures will be useful in determining the significance of this finding.

BODY OF REPORT

Introduction

A large body of scientific evidence has established that particulate air pollution has detrimental cardiopulmonary effects on human health. Multiple studies have identified particles from motor vehicle combustion engines as having particularly important adverse health effects. Accordingly, regulatory and monitoring policies have been established to limit particulate matter (PM) levels in the ambient air. These air quality standards have been based on yearly or daily averages. However, previous studies have illustrated that short-term exposure to some types of PM can lead to adverse health effects. Thus, there is a need for increased understanding of how ambient PM may impact human health over short exposure time periods.

Epidemiology studies have observed strong associations between particulate matter (PM) and cardiopulmonary health outcomes with PM implicated as a cause of increased human morbidity and mortality. With regard to respiratory health, cough, bronchitis, asthma, and chronic obstructive pulmonary disease are all associated with elevated particle levels. There is little doubt that particulate pollutants can exacerbate allergy and inflammation.[1,2] In addition, recent studies suggest that PM levels may also affect asthma and allergy prevalence.[2] In an urban setting such as the Los Angeles Basin, particles generated by vehicular traffic are thought to be important risk factors. An important contributor to PM2.5 (particles with an aerodynamic diameter less than 2.5 microns) from mobile sources is diesel exhaust particles (DEP). The ability of DEP to modulate the immune system has now been firmly established in human, animal and in vitro models.[3,4] Over a decade ago, studies at our institution and others demonstrated that DEP could modulate the immune system in the human upper airway. Similar studies have now been performed using diesel inhalation and have confirmed many of these effects in the lower airways as well[5-12]. Murine models have confirmed that DEP can turn a minimal inflammatory response into a robust one.[13-16] Thus, DEP is the best characterized of all the components of ambient air and is established as a model particulate pollutant. Although 80% of DEP is composed of particles in the 0.1-0.5 µm ranges, it is also an important source of ultrafine (< 0.1 µm) particles in ambient air.[17] DEP is a product of incomplete combustion and contains hundreds of chemical components including polycyclic aromatic hydrocarbons (PAH) that can modify the immune system.[18-20] Over the last 10 years we have gained a wealth of knowledge on the effects of PM on airway responses by the use of this model pollutant and a human nasal provocation model.[21-30] These studies have established that DEP can both induce and exacerbate *in vivo* allergic and inflammatory responses. Human atopic volunteers are sprayed intranasally with up to 0.3 mg DEP. This has been calculated as equivalent to 40 hours cumulative ambient exposure in Los Angeles, [31] a high dose but one that can be encountered as a bolus in certain occupational or everyday settings (such as waiting at a bus stop). Murine models have confirmed these effects and shown that DEP can enhance airway resistance and hyperreactivity.[13-16]

Far fewer studies have examined the effects of PM in the human lower airway though this is an area of active research. Diesel inhalation has been employed as a model of airway responses to PM. Researchers have shown that experimental exposure (typically with 300 µg/m^3 DEP) of non-allergic, non-asthmatic healthy subjects to diesel exhaust results in an increase in inflammatory cells, predominantly neutrophils and lymphocytes, in bronchial biopsies, BAL or

sputum[5-9] Studies using 200 µg/m^3 DEP without gases have recorded similar increases in neutrophil numbers in sputum, however, no increase in other cell types. [32]

Recent studies have shown that at lower concentrations (2 hour exposure at 100 µg/m^3 DE), healthy subjects demonstrated only mild bronchoconstriction.[33] In contrast the same authors reported that at higher DEP concentrations (1 hour at 300 µg/m^3), mild asthmatics have increased airway resistance and hyperreactivity.[12] Conversely, a direct comparison of healthy and mild asthmatics at the lower concentration (100 µg/m^3) showed differential effects of DE on cellular and cytokine responses between the two populations but no significant effect of DE on physiological measurements of airway inflammation.[11] Careful examination of the results presented showed that there was a wide inter-individual variation in responses and that at least two asthmatic individual made robust responses for all parameters. Thus, discrepancies in study findings may be due to the fact that airway responses are only apparent in a subset of subjects.

While DEP is an important constituent of concentrated air particles (CAPS), specific investigations with CAPS exposure have been more limited. A series of controlled human exposure studies with CAPS in the fine ($PM_{2.5}$), coarse ($PM_{10-2.5}$), and ultrafine ($PM_{0.1}$) size ranges at LAREI has so far failed to show convincing effects on the respiratory tract, in healthy and asthmatic adult volunteer groups exposed under conditions simulating "realistic worst case" ambient PM exposures.[34-36] Slight cardiac electrophysiologic and blood biochemical changes have been observed in these groups undergoing controlled CAPS exposures supporting the concept that the effects of PM reach beyond the respiratory tract and may induce reduced heart rate variability, increased blood coagulability, or systemic inflammatory responses. However, specific findings concerning these systemic effects have been inconsistent between studies. Several other investigators have observed similar results. Some data has shown that CAPS and other contributors to PM such as fly ash[37] also have the potential to alter immune function. Human studies demonstrate a large inter-individual variation in responsiveness to these particles, but the basis of this responsiveness is largely unknown and an important underserved area of investigation.

In order to identify potentially susceptible individuals, one must understand the mechanisms that underlie the deleterious effects of PM. In vitro and in vivo models have principally used DE or DEP as models of PM and shown that these particles and their constituent chemicals have multiple effects on several cell types. Bronchial epithelial cells,[38-43] alveolar macrophages,[44] mast cells and basophils, [30, 45, 46] eosinophils, and lymphocytes[47,48] are affected by DEP exposure suggesting a common mechanistic pathway. Consequently, research has focused on induction of cellular oxidative stress either directly or indirectly by chemical compounds present on the surface of the particles.[49,50] Murine and in vitro models have revealed a role for O_2^- generation in PM-induced inflammation [51-53] and many have shown that pre-treatment of mice or cells with antioxidants can block PM inflammatory effects[54]. PM can contain oxidant and pre-oxidant chemicals that react together or with gaseous oxidant pollutants (such as ozone) to form $ONOO^-$, $^{\cdot}OH$ and H_2O_2[55]. Inflammatory cells stimulated by these oxidants in turn produce reactive oxygen species (ROS). Measurements of oxidative stress are elevated in the airways after PM exposure.[8] Oxidative stress has multiple consequences on the airways including increased airspace epithelial permeability[56], mucus secretion,[57] and airway endothelium injury.[58] ROS can oxidize membrane phospholipids by lipid peroxidation, causing the production of lipid peroxides which impair

membrane function, inactivate membrane-bound receptors and enzymes, and increase tissue permeability.[55] Another important effect of oxidative stress in the airways is its ability to activate redox-sensitive transcription factors (e.g. NF-κB and AP-1), which regulate expression of many pro-inflammatory cytokines.

These studies have led to the concept and model of a hierarchical oxidative stress response to PM,[59,60] which postulates that inflammation occurs when high levels of oxidative stress overwhelm natural anti-oxidant responses. At low levels of oxidative stress, oxidant chemicals and the resultant ROS can be conjugated and eliminated by Phase II enzymes. Phase II enzyme genes are induced by oxidative stress as they contain an antioxidant response element (ARE) in their 5' flanking region[61] and are a primary defense for reducing or eliminating oxidative stress in mammals.[62] When this Phase II enzyme response fails or when higher doses of PM induce greater oxidative stress levels, increased activation of redox-sensitive transcription factors induce pro-inflammatory genes and increased inflammation commences. Using proteomics, Li et al have verified this model for the effects of DEP on macrophages. Additionally, it should be recognized that inflammation *per se* is an oxidative event.[63] Oxidant pollutants such as DEP can therefore have a dual effect by increasing oxidative stress directly via the induction of ROS by the chemicals they contain, and indirectly by causing enhanced inflammation and thus additional ROS generation.

By way of background, our research group has performed hundreds of DEP nasal challenges over the past decade and has demonstrated the enhancing effect of DEP on allergic inflammation. However, the inter-individual variation in these responses has been very large. DEP can enhance allergen-specific IgE by over 50-fold in some individuals, but in others will have no effect. This feature has also been reported for PM and other gaseous pollutants. For example, decreased pulmonary function, rhinitis symptoms and enhanced nasal airway resistance following secondhand smoke exposure occurs only in a subpopulation (33%) of asthmatics.[64,65] The first step in understanding susceptibility to PM is determining whether this is an intrinsic trait or due to extrinsic factors.[66] Previous work demonstrated that individuals responses to DEP were reproducible. Eighteen non-smoking atopic volunteers underwent nasal challenge with DEP; after at least 30 days, the procedure was replicated. This repeated challenge produced reproducible nasal responses for cell influx and cytokine production. DEP's ability to enhance inflammatory responses was highly reproducible within individuals suggesting that susceptibility to DEP's effects is an intrinsic trait. Similar results were seen in allergic endpoints (IgE, Th2 cytokines) when the experiment was repeated with combined allergen and DEP challenge.

Based on these findings, our group has been able to identify individuals with reproducibly robust or modest airway inflammatory responses to DEP exposure. This work has further identified one characteristic that can define a subgroup susceptible to DEP. As explained, Phase II enzymes are critical in protection against oxidative stress. Variations in the activity of a number of these human Phase II antioxidant enzymes have been identified that arise from polymorphisms in the coding genes. Previous work has shown that variation in key Phase II enzyme genes (GSTM1 and GSTP1) dictate differences in susceptibility to the inflammatory effects of DEP. In a crossover single blind study,[67] human subjects were challenged with either 0.3 mg DEP or placebo intranasally. After at least a month, the subjects were recalled and those previously exposed to placebo received DEP and vice versa. Cells from buccal scrapes were then genotyped

in a blinded fashion to determine which variants of the GSTM1 and GSTP1 genes each subject carried. Individuals with GSTM1 *null* and GSTP1 *ile/ile*, (forms of the genes that result in absent or reduced anti-oxidant responses) showed heightened inflammatory responses to DEP. Even with a small sample size, significant associations were observed between genotype and DEP-enhancement of inflammatory markers of cell influx, IL-8, and other inflammatory cytokines such as GM-CSF by DEP. This demonstrates the importance (penetrance) of these genetic polymorphisms in determining host response to DEP. These studies show that at least in the upper airways, one subgroup at increased risk for the inflammatory effects of diesel can be identified on the basis of genotype. These genes are expressed and play an identical role in both the upper and lower airway. Epidemiology studies have also implicated GSTM1 null as a risk factor for PM- and ozone-induced asthma[68,69].

In order to establish whether DEP effects are unique or can be replicated by other oxidant particulate pollutants, environmental tobacco smoke (ETS) has also been studied in similar human models. Inhalation challenges to ETS (2 hours, 5 cigarettes) were performed on allergic subjects. For all endpoints measured (e.g. antibody, cellular, cytokine and histamine production and release), ETS replicated the ability of DEP to augment inflammation and allergen-induced changes. To determine whether responsiveness to the adjuvant effects of ETS was related to responsiveness to DEP, nineteen ragweed-allergic subjects underwent nasal allergen challenge, ETS exposure/allergen challenge, and DEP/allergen challenge. Each challenge was spaced at least 3 months apart. Ragweed-specific IgE was measured in nasal lavages performed 4 days after the challenge and the adjuvant effect of each pollutant was examined.

A highly significant correlation was observed between responsiveness to the adjuvant effects of the two pollutants. Those individuals who were refractory to the effects of DEP were also non-responsive to ETS, while those in whom ETS had a profound effect on IgE enhancement also tended to show augmented IgE responses by DEP. Similar results were observed for cytokine and cellular responses to DEP or ETS alone or with allergen. Genotype analysis again showed that individuals with the GSTM1 *null* polymorphism had significantly stronger inflammatory responses than those with fully functional versions of the gene.

Since publication on the role of GSTM1 on DEP-susceptibility, much attention has been focused on the role of this polymorphism in other PM- related effects. Several studies have now shown that the GSTM1 *null* polymorphism is an important risk factor for developing asthma in children of smoking parents.[70-72] A recent study showed that a 10 ug/m^3 increase in PM2.5 during the 48 hours prior to a measurement of heart rate variability was associated with a 35% decrease in this value but only in subjects who were GSTM1 null and not in those with GSTM1 present.[73] Once again, these studies show that analysis of PM effects on an unselected population is likely to overlook important effects is susceptible subgroups. Thus, evidence suggests that other particulate pollutants operate via similar mechanisms to diesel exhaust and that GSTM1 is a marker of susceptibility for other components of PM. Critically, the role of GSTM1 has not been studied directly on CAPS exposures.

Nasal challenge with DEP and other particles is a convenient and technically simple model in which particle effects in the upper airway can be studied in a large number of subjects with relative ease. A more physiologically relevant model is that of inhalation using exposure

chambers to examine effects of diesel exhaust or CAPS on defined phases of the human inflammatory and allergic response in the lower airways.

Short-term exposure to PM can cause airway inflammation. Murine, cellular and human studies all confirm this fact, though human studies that have directly tested short-term PM exposure on human subjects have reported only modest, subtle changes in disease markers likely because of the great heterogeneity in responses observed. A few studies show that marked significant changes do occur but only in some subjects. It is clear that responses to PM are heterogeneous and depend largely on intrinsic factors of the individual. Thus, it becomes important to study individuals with risk factors identified as conferring likelihood of increased susceptibility or responsiveness to PM. Based on available data we have selected the two most prominent "susceptibility factors" (GSTM1 null genotype and asthma) to study. These susceptibility factors, specific genotype (presence of the *GSTM1* null polymorphism) and underlying pulmonary disease (asthma) are very common (50% and 8%[74] respectively). By investigating these "at-risk" populations specifically, it is possible to determine the impact of PM on a large segment of the population which is potentially most vulnerable to the adverse health effects of air pollution. Such studies may allow future air quality regulations to not only protect the health of the general public, but to additionally protect the health of subpopulations at significantly greater risk. It is well-understood that significant sections of the population may be more vulnerable to PM health effects than others. Identification and protection of these people is of enormous public health importance. Additionally, as evident from this brief review of the literature, there is considerable data on the effects of DE and DEP on inflammation but less is known regarding the effects of CAPS. Available in vitro and in vivo data support the oxidative and inflammatory effects of CAPS, however there are a paucity of controlled human exposure studies examining respiratory and systemic effects of CAPS in potentially susceptible populations.[75]

The primary objective of this study is to test the hypothesis that individuals with certain 'susceptibility factors' will have heightened inflammatory and airway responses to exposure to concentrated ambient particles (CAPS). An additional central question in this study is the role of these susceptibility factors in determining responses to CAPS specifically, given the paucity of data for this type of particle exposure.

We aimed to test the concept that concentrated air particles (CAPS) induce inflammation preferentially in an identifiable subpopulation. The susceptibility factors, chosen based on previous findings, are genotype (presence of the *GSTM1* null polymorphism) and underlying pulmonary disease (asthma). We compared responses in these likely susceptible individuals to those without any corresponding risk factors, by studying the physiologic and inflammatory effects of short-term PM exposure. This inflammatory response is believed to be the primary mechanism responsible for the deleterious cardiovascular and respiratory effects of PM. The study design for the project is unique in examining a potentially genetically-susceptible subpopulation in a prospective manner using controlled respiratory CAPS exposure.

In order to test the hypothesis, subjects were recruited based on GSTM1 genotype and asthma status. For comparison, we enrolled three distinct groups for the exposure protocol: GSTM1-null asthmatics, GSTM1-present asthmatics, and GSTM1-present healthy subjects. These groups are believed to be at "high", "medium", and "low" risk respectively for the adverse effects of

PM. Enrolled individuals were exposed to physiologically relevant concentrations of fine CAPS or filtered air. Subsequently, we compared the resultant inflammatory and airway responses in subjects of different risk categories. Cardiovascular measurements were collected by 24-hour Holter monitor as well. Particles were characterized for CAPS and FA conditions. Inflammatory outcomes and physiologic measurements were analyzed for comparisons between groups.

Materials and Methods

We conducted a single-blind randomized crossover study of controlled exposure to filtered air (FA), and to concentrated ambient fine particles (CAPS), in 10 GSTM1 null mild-moderate asthmatics, 10 GSTM1 positive mild-moderate asthmatics, and 10 GSTM1 positive healthy subjects. Exposures lasted two hours, with submaximal exercise (approximately tripling resting ventilation) for 15 min of every half-hour. The target CAPS concentration was 200 $\mu g/m^3$. The exposure equipment and procedures were the same as in previous concentrated-fine-particle exposure studies using non-genotyped mild asthmatics[36] and are described in detail in the attached Appendix. The overall schema for the exposure schedule is represented in Table 1, though the FA or CAPS exposure order was randomized. The protocol for specific measurement of subject responses during each exposure period is detailed in Table 2.

Table 1: Schema for schedule for exposures

Day 0	screening visit
Day 14	exposure to 200 ug/m^3 CAPS*
Day 15	follow-up visit
Day 28	exposure to filtered air*
Day 29	follow-up visit
* Order of exposure to CAPS and FA randomized for each subject	

All human subject screening examinations, experimental exposures, measurements of clinical and physiologic response, and biological specimen collections were performed in the Environmental Health Service of Los Amigos Research and Education Institute (LAREI), located within Rancho Los Amigos National Rehabilitation Center (RLANRC) in Downey, California. Genotyping and analyses of sputum and blood samples were done at the Division of Clinical Immunology/Allergy at UCLA. Holter electrocardiographic recordings were sent to the National Human Exposure and Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, North Carolina for analysis.

Human Subjects

All human subject procedures were approved by the appropriate institutional review boards at LAREI and UCLA. Subjects were recruited through LAREI by word of mouth, invitations to eligible previous volunteers, and local media advertisements. Interested subjects completed the informed consent process and were then screened for study eligibility.

Subjects were screened to identify and enroll 10 subjects in each of three specific groups:

1) Mild-Moderate persistent asthmatics with the GSTM1 *null* genotype.
2) Mild-Moderate persistent asthmatics with the GSTM1 *present* genotype.
3) Healthy non-asthmatics with functional forms of GSTM1 *present*.

Table 2. Exposure and Subject Evaluation Protocol

Clock Time	Time from start of Exposure	Activity (in sequence)
07:30	-1:30	Subject arrives in the laboratory and rests in clean air. Symptom score sheet completed. Initiation of Holter ECG, telemetry, pulse oximetry. Vital signs (heart rate, blood pressure, respiratory rate, SaO_2) and cardiopulmonary physical examination. 12-lead ECG at rest.
08:20	-0:40	Venous blood drawing (20 ml). Exhaled CO and NO measurement. Nasal lavage
08:30	-0:30	Pre-exposure spirometry. Urine collection
09:00	0:00	Begin 2-hr exposure with intermittent exercise. Symptom score sheet completed every 15 min. SaO2 measured every 15 min Minute ventilation measured during the end of final rest and exercise periods
11:00	2:00	End of exposure. Subject rests in clean air. Vital signs, exhaled CO and NO measurement, spirometry.
12:00	3:00	Symptom score sheet.
12:30	3:30	Methacholine bronchoprovocation test with spirometry.
13:00	4:00	Symptom score sheet. Spirometry.
13:10	4:10	Subject leaves laboratory with diary to record overnight symptoms.
[Day 2]		
09:00	24:00	Subject arrives in the laboratory and rests in clean air. Diary and Holter recording collected. Symptom score sheet. Vital signs and cardiopulmonary examination.
09:30	24:30	Venous blood drawing (20 ml). Urine collection
09:45	24:45	Spirometry. Exhaled CO and NO measurement.
10:00	25:00	Nasal lavage. Sputum induction. Spirometry.
10:45	25:45	Subject leaves laboratory.

As previously discussed, these three groups were selected based on data suggesting they represent gradients of susceptibility to the pro-inflammatory effects of PM and biologic differences in PM-response between the three groups should be apparent.

Asthmatics

Subjects with mild-moderate asthma (Step 2-3 NAEPP classification) were enrolled in the study. Asthma severity is believed to be an important risk factor for PM responsiveness. It would be informative to study subjects with more severe asthma symptoms. However, inclusion of

subjects with more severe asthma was not feasible due to bioethical, safety, and scientific considerations. By definition, 'moderate' asthmatics present with daily symptoms and according to published consensus guidelines require minimum medication of low-dose inhaled corticosteroids and long-acting inhaled beta2-agonists or medium-dose inhaled corticosteroids. As inflammatory biomarkers are a primary outcome for the study and corticosteroids are anti-inflammatory, it was necessary to exclude any subject using inhaled or systemic corticosteroid therapy. Withholding recommended asthma medication from subjects for the purposes of study participation was unethical. However, a large proportion of moderate asthmatics do not receive inhaled corticosteroid therapy. While we did not diagnose or prescribe treatment in this study, when we encountered subjects with clear evidence of moderate asthma, the appropriate action from an ethical standpoint was to provide them with education on asthma treatment guidelines and advise them to consult their personal physician. In addition, many moderate asthmatics have PEF or FEV1 lower than 70%. A significant reduction in lung function with PM exposure in such individuals could require emergency medication or hospitalization so safety concerns were of utmost importance.

Within the category of mild asthmatics there are individuals of differing severity. For example, in this category daily symptoms can occur as infrequently as twice a week or as frequently as six times a week. We aimed to recruit subjects who were at the upper end of the mild spectrum i.e. mild-moderate. A recommended treatment for these patients is also low dose corticosteroids, however, NAEPP guidelines state that "alternative treatment (listed alphabetically): cromolyn, leukotriene modifier, or nedocromil," can be used. Many mild-moderate subjects are not on corticosteroids and in consultation with their physician decide to use medication only on an "as-needed basis". Previous studies on the effect of leukotriene inhibitors in attenuating acute airway cellular inflammation (as opposed to the late-phase response) has shown that they are ineffective. (Details of these studies cannot be presented here as this is considered privileged data.) Our selection criteria were therefore aimed to enroll subjects with asthma symptoms but who did not require or use inhaled corticosteroids.

Selection criteria

Group 1 subjects fulfilled the following criteria:

a. Step 2 asthmatics according to the NAEPP guidelines for the Diagnosis and Management of asthma but otherwise healthy.
b. GSTM1 null polymorphism as determined by RFLP from cells obtained by buccal scrape at screening.
c. Screening FEV1 >70% predicted (per lab screening).
d. Daily asthma symptoms 4-6 times a week
e. Positive methacholine bronchoprovocation, i.e., 15% decrease in FEV_1 with <8 mg/ml methacholine inhalation (per lab screening).

Group 2 subjects fulfilled the following criteria:

a. Step 2 asthmatics according to the NAEPP guidelines for the Diagnosis and Management of asthma but otherwise healthy.
b. GSTM1 positive polymorphism as determined by RFLP from cells obtained by buccal scrape at screening.

c. Screening FEV1 >70% predicted (per lab screening).
d. Daily asthma symptoms 4-6 times a week
e. Positive methacholine bronchoprovocation, i.e., 15% decrease in FEV_1 with <8 mg/ml methacholine inhalation (per lab screening).

Group 3 subjects fulfilled the following criteria:

a. Never diagnosed with asthma.
b. GSTM1 positive polymorphism as determined by RFLP
c. Normal screening FEV_1 of >80% predicted.
d. Negative methacholine bronchoprovocation (no 20% fall in FEV_1 with <25 mg/ml methacholine).

All study subjects fulfilled the following criteria:

a. 18 years of age or older
b. Nonsmoker or ex-smoker (stopped >2 yr prior to study entry)
c. Absence of any exclusionary criteria:
    1. significant disease(s) or condition(s) other than specified asthma which might affect study results or contraindicate additional stress on the cardiorespiratory system
    2. pregnancy
    3. inability to perform exercise
    4. inability to provide informed consent

Study Procedures

Screening Visit

Eligibility and asthmatic status was determined during the screening visit. During this visit subjects underwent: a medical history; cardiopulmonary physical examination; urine pregnancy test (applicable only to women of child-bearing potential); resting electrocardiogram (ECG); lung function testing (spirometry); and a submaximal exercise test on a stationary bicycle (tripling resting ventilation). In addition, methacholine challenge testing was performed to determine the amount needed to record a 20% fall in FEV_1. Buccal cells were collected for genotyping.

Subjects arranged their own transportation to LAREI for screening and exposure days. One unavoidable confounder was background air pollutant exposure en route to LAREI. It was beyond the budgetary and scientific scope of the project to monitor personal exposure on days before the visits. Subjects were encouraged to use surface roads to travel to LAREI and to avoid exposure to smoke and traffic fumes as much as possible. Subjects were asked to fill out a symptom score diary for the 48 hrs prior to exposure and for 24 hours post. The diary included a component designed to identify any defined incidental air pollutant exposure during the course of the study protocol. Nevertheless, it is important to note that previous studies show responses to PM exposures occur regardless and independent of background PM levels.

Eligible subjects were assigned to one of the 3 study groups based on presence or absence of asthma diagnosis and GSTM genotype. Within the groups, subjects were randomized to one of two exposure sequences, which received either control or 200 ug/m^3 CAPS first. With 10

subjects per group, randomization was matched for each group so that 5 individuals in each group were assigned to each of the different exposure sequences.

Study Visits

Each subject completed study visits per Table 1 with exposure and measurement schedules as outlined in Table 2. The protocol included a total of 5 visits: 1 screening visit, 2 exposure days, and 2 follow up visits 1-day post-exposure. Experimental exposures were separated by at least 2 weeks. A 2-week washout period was chosen based on previous studies using CAPS and DE inhalation exposures.[21, 32] A 1-week washout period appears sufficient to ensure no "spill-over" effect from one methacholine challenge to another.[76]

Experimental Exposures

The schedule for each experimental exposure day was identical regardless of exposure given. Because circadian variation may influence some experimental endpoints, all exposures for a given individual were started at the same time of day (within 30 min). Upon completion of exposures, subjects received $beta_2$-agonist as needed and were free to leave when their FEV_1 had stabilized. Asthmatic subjects were provided with peak flow meters and instructed to document late reactions and symptom diaries and to take beta-agonist medication if needed. CAPS atmospheres were invisible and were not readily distinguishable from FA by either subjects or by staff members making health measurements. Separate staff members were responsible for exposure atmosphere generation and monitoring.

CAPS Exposure:

Each subject was exposed in a whole-body chamber to CAPS (PM2.5) at a nominal concentration of 200 $\mu g/m^3$ monitored in real time by a nephelometer and controlled by diluting the output of the ambient fine particle concentrator with varying amounts of filtered air. The particle concentrator, interfaced to a single-person exposure chamber (volume ~4 m^3) containing a foot-crank exercise ergometer, was used to simulate "worst-case" ambient exposures over 2-hour periods: subjects exercised moderately for 15 min of each half hour reaching ventilation rates approximately 3 times resting. This system has been previously used safely and effectively during exposure of asthmatics to concentrated air particles.[34] Additional technical details of the CAPS exposure system were originally published as part of a 2003 Health Effects Institute Report which is included as Appendix 1 to this report. Briefly, the concentrator itself follows the original design reported by Sioutas et al.,[77] and resembles that used by the EPA in their human studies. The outdoor ambient air used to obtain concentrated particles for exposure is drawn from above the roof of the laboratory, about 4 m above grade. Ambient particles are concentrated as much as 9 times. Important contributors to ambient PM at the laboratory location include the southern Los Angeles County background pollution, locally heavy surface-street traffic, the diesel-truck-heavy I-710 freeway about one mile west, and the port complex about 10 miles south. Previous work has characterized this neighborhood's pollution[78,79] and the fine CAPS exposure atmospheres[80, 81] in some detail. Nitrate, organic carbon, sulfate, and elemental carbon are major constituents of the fine CAPS. Particle size distribution measurements were performed with a micro-orifice uniform-deposit impactor (MOUDI) to determine the contribution of ultrafine particles to CAPS.

Filtered Air Exposure:
Filtered air (FA) exposure was used as a control arm in the study. All FA exposures were performed in the same chamber. Subjects were exposed as above except that ambient air was filtered by HEPA particle filtration.

Carbon monoxide, nitrogen oxides, sulfur dioxide, and ozone levels were monitored in incoming ambient air upstream of the particle concentrator during FA and CAPS exposures. Prior testing has shown little difference between ambient and in-chamber measurements of gases.

Exposure Characterization
A nephelometer was used to monitor particle mass concentration vs. time during CAPS exposures. For gases, a carbon monoxide infrared analyzer and a chemilluminescence ambient NO/NO_2 analyzer (Advanced Air pollution) was used for real-time accurate measurement of their respective gaseous levels. Other samples were periodically withdrawn and analyzed for those compounds for which continuous monitors are not available. During CAPS exposures, it was not possible to measure levels of other gases (e.g. ozone, SO_2, NO_x) inside the chamber due to issues of space. Instead, since the concentrator does not exclude or concentrate gases, ambient concentrations were measured as a proxy.

In each exposure, low-volume filter samples were obtained by drawing air onto separate Teflon and quartz filters. Quartz filters were used to determine elemental and organic carbon by the method of Fung.[82]

CAPS samples were collected to analyze PAH content. Studies performed at UCLA show that there is a very close correlation between the inflammatory properties of particles and the amount of PAH contained on the particles.[83] Use of filter samples to measure other particle characteristics including elemental content was performed as possible within technical limitations.

<u>Assessment of Biological End Points:</u>

Pulmonary measurements
Nonspecific bronchial reactivity:
Methacholine challenge testing was performed 90 minutes after each exposure to measure Non-specific bronchial hyperresponsiveness (NSBH), a typical mark of asthma. Methacholine challenge is considered the "gold standard" to measure changes in NSBH.[84,85] We followed the method of Chai and coworkers,[84] as used successfully in previously exposure studies.[34,76,86] doubling doses of methacholine were generated and delivered aerosolized through a nebulizer connected to a dosimeter. The subject inspired from the nebulizer and a solenoid valve in the dosimeter opened for a preset time allowing the calibrated delivery of aerosol for five breaths for each concentration. Measurement of forced expired volume in one second (FEV_1) was performed 1.5 minutes after each dose, and repeated at 3 minutes to document a sustained drop. The amount of methacholine required to effect a 20% fall in FEV_1 was recorded. A maximal methacholine concentration of 10 mg/mL was administered.

Spirometry:
Spirometry for forced expiratory lung function measurements (FVC, FEV_1) were performed with a Jaeger MasterScreen IOS pneumotachograph system (Viasys Inc., Yorba Linda, CA), certified to meet American Thoracic Society (ATS) standards of accuracy, calibrated with a 3.00-liter volumetric syringe by the manufacturer's procedure at the beginning of each day's testing. Spirometry was performed at the screening visit and immediately prior to and after exposures as well as 24 hours later. Normal spirometric data was based on reference standards published by Morris and coworkers.[87]

Exhaled Nitric Oxide and Carbon Monoxide:
Exhaled nitric oxide (FeNO) was measured with a Sievers 280i chemiluminescent analyzer system (GE Analytical Instruments, Boulder, CO), calibrated daily with a Sievers zero-air filter and a certified nitric oxide span gas (Scott-Marrin Inc., Riverside, CA). Measurements were performed according to American Thoracic Society (1999, 2005) recommended procedures, at 50 mL/sec expiratory flow after a vital-capacity inspiration of unfiltered room air. Ambient nitric oxide concentration during the test session was recorded, in order to determine whether it had statistically significant influence on the exhaled concentration. Exhaled CO was measured with a Bedfont EC50 Micro III Smokerlyzer (Bedfont Scientific, Rochester, Kent, UK), calibrated at the beginning of each testing day with certified zero and span gases (Air Liquide Inc., Long Beach, CA). With the instrument in continuous monitoring mode, ambient CO concentration was recorded initially, then the subject held his/her breath for 20 sec before exhaling into the sampling chamber.

Cardiovascular endpoints
Changes in cardiovascular/electrical activity of the heart were measured in subjects by 24 hour monitoring using Mortara Instrument Holter monitors (Model H12 Plus-AAA). Monitoring started upon arrival for the exposure day and lasted until the follow-up day (approximately 24 hours). Data gathered by the monitors was analyzed for various indices of heart rate variability, S-T voltage, and repolarization at the University of North Carolina in collaboration with the EPA, Human Studies Division.

Inflammatory Biomarkers in Sputum and Nasal Lavage
Cell influx, pro-inflammatory, and pro-allergic cytokines:
Sputum samples were collected at baseline, and 24 hours post each exposure (3 collections per subject). Sputum was processed by measuring the total volume of sputum and adding an equal volume of 0.1% dithiothrietol (Sputalysin 10%, Behring Diagnostics) with subsequent homogenization by gentle vortex mixing and a shaking water bath at 37^0C for 30 min. After adding a fixed volume of PBS, a total cell count was performed. The sample was then centrifuged, the cell pellet used for differential cell staining, and the supernatant used for fluid phase measurement assays. Per laboratory standard operating procedures, samples were frozen at -80^o C between collection and batched analysis. No interim analysis on samples was performed. Differential cell counts (based on >=200 cells/sample) included percentages of monocytes, lymphocytes, polymorphonuclear leukocytes (PMNs), and eosinophils by Giemsa staining, as well as eosinophils by Wright staining. The following substances were assayed in the fluid phase: immunoglobulins IgG, IgG4, IgA, IgM, and IgE; interleukins IL-4, IL-5, and IL-8; granulocyte/macrophage colony-stimulating factor (GMCSF); interferon-gamma (IFN-γ), tumor

necrosis factor alpha (TNF-alpha). Most assays employed monoclonal antibodies commercially purchased or manufactured at the UCLA laboratory, according to manufacturers' instructions or previously reported protocols.

Nasal lavage samples were collected pre-, 2, and 24 hours post each exposure (6 collections per subject). Samples were spun at 300g and total cell count was performed on cells recovered. Then an aliquot of 5 x 10^4 cells was spun onto microscope slides by Cytospin® and differential cell counts performed by modified Giemsa staining. The supernatant was concentrated and used for fluid phase measurement assays. The following substances were assayed in the nasal lavage supernatant: IgG, IgG4, IgA, IgM, and IgE; IL-4, IL-5, and IL-8; IFN-γ, TNF-alpha.

Polystyrene microtitre plates (Corning, Lowell, MA) were used for all assays. The plates were washed with phosphate-buffered saline (PBS/tween) and blocked with 1% BSA-PBS. Assay diluent used was 1% BSA-PBS. Human cytokines IL-4, IL-5, IL-8, IFN-γ, GMCSF, and TNF-α were measured from sputum and NL supernatants using commercial BD Opt EIA kits (BD Biosciences, San Diego, CA) following the manufacturers' instructions. The sensitivity of the commercial ELISA kits was 0.2 pg/ml. Antibody isotype (IgE, IgG, IgG4, IgM, and IgA) levels in supernatants obtained from sputum or NL samples were measured by isotype-specific enzyme-linked immunosorbent assays (ELISAs). Briefly, plates were coated overnight with the appropriate anti-Ig antibody at 2ug/ml. For total IgE, human IgE monoclonal antibody (4.15 and 7.12) and standard (WT IgE) produced in the UCLA Hart and Louise Lyon Immunology Laboratory were used with detection antibody at 1ug/ml (KPL, Gaithersburg, MD). For human IgA, the standard curve employed started at 10µg/ml, and detection antibody was used at 1ug/ml (Caltag, Burlingame, CA). Human IgG and IgG4 were measured using a standard starting at 10µg/ml and 1µg/ml respectively while human IgM was measured using a standard starting at 1 µg/ml (Sigma, St. Louis, MO). Detection antibody for IgG and IgM were used at 1/1000 dilution (Biosource, Camarillo, CA) and IgG4 at 1/1000 dilution (BD Biosciences, San Diego, CA). Sensitivity of antibody assays was 1 ng/ml.

Systemic inflammatory biomarkers

Blood factors:
Venous blood samples were conventionally processed. Serum samples were frozen at -80^o C between collection and analysis. Measurements of factors in serum were performed using commercially available kits as denoted: C reactive protein (Hyphen BioMed, Neuville-sur-Oise France, sensitivity-5.1ng/ml), Factor VII (Hyphen BioMed, Neuville-sur-Oise France, sensitivity 5%), von Willebrand factor (Hyphen BioMed, Neuville-sur-Oise France, sensitivity 7.8%), fibrinogen (Innovative Research, Novi, Michigan, sensitivity-3.125ng/ml), IL-8 (BD Biosciences, San Diego, CA, sensitivity 0.2 pg/ml). Additionally, serum antibodies levels (IgE, IgG, IgG4, IgA, and IgM) were measured using methodology described above to detect any effects of CAPS exposure on systemic antibody responses.

Urine:
8-isoprostane levels were measured in the urine of subjects following exposures. Commercially available kits (Northwest Life Science Specialties, Vancouver, WA, sensitivity-0.05ng/ml) were used following manufacturer's instructions.

Description of Other Procedures

Sputum

Standardized techniques to induce sputum were used for the protocol. These have previously been used to detect acute airway inflammation following controlled exposures to pollutants [88, 89] Before the procedure the subjects inhaled a $beta_2$-agonist (albuterol, four puffs, 360 µg) to block bronchoconstriction. None of the measurements in sputum (cell counts, antibody, cytokines) are altered by pretreatment with $beta_2$-agonist or by methacholine challenge.[90-92] Sputum induction immediately prior to exposure may modify lung function and so this was not performed. The subject inhaled ultrasonically nebulized 3% sterile saline solution for 20 min. Every 2 min the subject actively coughed and expectorated saliva and sputum into separate sterile specimen containers to reduce contamination of sputum with saliva.[93] FEV_1 was measured by spirometry for safety.

Nasal Lavage

Nasal lavage was performed using standard collection techniques. Each complete lavage consisted of 10 mL normal saline administered in up to 5 mL aliquots to each nostril. The subject held their breath for approximately ten seconds while 5 mL of normal saline was placed via pipette into one nostril. After ten seconds the lavage fluid was collected. The process was repeated for the remaining nostril.

Buccal cell collection and genotyping

Buccal scrapes were performed during the screening visit and recovered cells used to extract DNA and genotype for GSTM1 polymorphisms. The buccal scrape was performed by gently scraping the inside of the mouth around the cheek with a toothbrush. Genotyping for GSTM1 was performed on a routine basis at the Division of Clinical Immunology and Allergy at UCLA.

Symptom score sheet and diary

Subjects completed a symptom score sheet pre-exposure and every 15 minutes during the exposure, as well as immediately post, 1 hr post, 2 hr post, and day 2 post-exposures. A total symptom score and subtotals for respiratory, cardiovascular, and miscellaneous (nonspecific) symptoms were calculated for each time the diary was filled out, using a slight modification of a procedure previously reported.[36] The symptom scoring procedure is demonstrated in Table 3. Average score changes, relative to the pre-exposure value, were calculated for the exposure period and for the follow-up period (immediately post-exposure through day 2). Thus, a negative score change would represent overall symptom improvement, and a positive change would represent worsening. The subjects also kept a symptom diary for the 48 hrs prior to exposure and for 24 hours post. This diary was primarily used for safety. The subjects reported any symptoms in several categories including cardiac, pulmonary, general well-being and medication use.

Table 3. Symptom Scoring

Symptom Category		
Respiratory	**Cardiovascular**	**Miscellaneous**
Cough	faintness/dizziness	headache
Sputum	fast heartbeat	fatigue
substernal soreness	irregular heartbeat	stomach upset
shortness of breath	pain related to heart	eye irritation
Wheeze		other
chest tightness		
pain related to lungs		
sore throat		
nasal discharge/congestion		

Scoring for Each Symptom at Each Time of Recording		
0 Not present		
1 Minimal		
2 Mild		
3 Moderate		
4 Severe		
5 Incapacitating		

Other Physiologic Measurements
Blood pressure was measured with an automated sphygmomanometer (Sunbeam Inc., Hattiesburg, MS) and arterial oxygen saturation with a fingertip pulse oximeter (Nellcor Inc., Boulder, CO), both employing internal calibration standards.

Quality Control Procedures

Instruments were calibrated in the appropriate laboratory at UCLA, LAREI, or CE-CERT before and during the study.

Gravimetric Analyses: The continuous particle monitoring instruments (SEMS, nephelometer) can be calibrated only by the makers. Thus, comparison of their mean readings during each exposure period with the concurrent integrated filter samples provides the necessary practical assurance that these instruments remain stable over time. Filter samples for gravimetric analysis are collected either in multiple size ranges (by MOUDI) or as a single sample by low-volume collection. All filters are conditioned for 24 hr to a standard temperature and humidity before weighing on an electronic microbalance. At each weighing session, a standard unexposed filter and a standard 100-mg weight are measured to verify balance performance. Records for these gravimetric standards have been maintained continuously for >10 years. The balance is tested and certified at 6-month intervals by an outside service firm.

SEMS: This instrument has been calibrated on two prior occasions at CE-CERT by their procedure using uniform polystyrene microspheres. Given reproducible results on those occasions, plus the above-mentioned continual checks versus gravimetric data, no new "factory" calibration of this instrument is required.

ELISA machine: This machine was calibrated daily by placing a reference substrate in a 96-well plate and comparing optical density readings at different wavelengths to reference values. In addition, each assay contains a reference positive control sample of known concentration.

API NO, NO_2, NOx Chemiluminescent analyzer: This instrument was calibrated with a commercial gas blend with a concentration of NO traceable to NIST monthly during the study.

Other: Pulmonary function test instruments were calibrated daily before subject testing by injections at multiple flow rates from a 3-liter volumetric syringe, following the manufacturer's recommended procedure.

Samples Documentation and Chain-of-Custody Procedures: All samples were assigned a code and chain-of-custody tracked. Logbooks were maintained at each site to document instrument calibrations, experimental procedures, and observations. Exposure filters were stored in Petri dishes and refrigerated. Filter samples were sent to the analytical laboratory for metal analysis while maintained chilled with ice.

Statistical Analyses

Each cytologic and immunologic variable was subjected to analysis of variance with repeated measures on subjects, the conventional statistical approach for laboratory studies with each subject exposed to both an experimental and a control atmosphere. We used SAS procedure MIXED (SAS Statistical Software, Cary, NC) to estimate effects of susceptibility group (healthy vs. asthmatic GSTM1-positive vs. asthmatic GSTM1-null), atmosphere (CAPS vs. FA), and time (pre-exposure baseline [B] vs. 2 hr post-exposure [P2] vs. 22 hr post-exposure [P22]). (Pre-exposure sputum sampling was not feasible, so a baseline measurement was taken during a separate laboratory visit with no exposure study, for comparison against P22 measurements.) Subject was treated as a random factor in these analyses; group, atmosphere, and time were treated as fixed factors. The MIXED procedure was used because it could handle unbalanced designs easily. Unbalance occurred for some variables because a few data were missing due to unsuccessful sample collection or problems in sample handling.

Selected variables were analyzed in more detail, by "advanced" statistical models more commonly used in large-scale drug trials. The advanced models tested effects of period (first vs. second vs. third laboratory visit), sequence (the particular order in which a subject experienced the different experimental conditions), and/or carryover (influence of a previous exposure on a current exposure, modeled as the period-by-atmosphere interaction) in addition to the effects tested in the aforementioned "conventional" statistical models. Although period effects were significant in some instances, conclusions from "conventional" and "advanced" models concerning the significance of CAPs effects were usually the same. Thus, "conventional" results are reported here, unless noted otherwise.

To assess the need for data transformation to normalize distributions, overall data distributions were examined for all variables, and distributions of residuals from "conventional" analytical models, broken down by levels of the repeated-measures factor, were examined for selected variables. For variables that were quantifiable in all cases, distributions were typically non-normal on the original scale but close to normal after log transformation. For samples with undetectable concentrations of some species, the value of each affected variable was estimated as one-half the lowest quantifiable measurement, to allow log-transformation. After transformation, distributions of those variables were improved but still non-normal. In general, data were analyzed both on the original scale and after log-transformation. Usually the statistical conclusions were similar either way. More details of statistical modeling are presented in the Results section.

Results

Human Subjects

Thirty-one subjects were enrolled and 30 subjects completed the study protocol. Table 4 contains the demographic data for subjects enrolled in the study protocol. One subject (2886) withdrew from the study protocol during the second exposure due to worsening of pre-existing gastrointestinal symptoms. In the investigator's judgment, these symptoms were unlikely to be related to the study procedures. The subject fully recovered within 48 hours, but did not complete the remaining protocol evaluations and thus was excluded from analysis. An additional subject was enrolled in her place.

No other adverse events were observed or recorded during the study. All other subjects completed the study protocol activities without complication.

Most asthmatics reported taking no asthma medications other than inhaled bronchodilators when needed; all denied taking inhaled or systemic corticosteroids which were study exclusion criteria. Thus, asthmatic subjects generally had mild disease.

The original objectives of the study included enrollment of atopic asthmatics as determined by allergy skin testing at the time of screening. This procedure was not integrated into the original study screening procedures at LAREI and therefore only allergy skin test results are not available for all subjects. Allergy skin test results available for some subjects from on-site testing included in previous study protocols are included in Table 4. All tested asthmatics had multiple unequivocally positive skin test responses, while tested healthy subjects were generally nonatopic with absent or minimal skin test reactions.

Table 4. Subject Characteristics

ID	Asthma	GSTM1	Age	Sex	Height	Weight	Ethn.*	Allergy†
2717	No	+	25	F	66	175	H	none detected
2723	No	+	19	F	64	131	W	[D]
2840	No	+	53	M	73	194	B	none detected
2843	No	+	26	M	69	218	H	none detected
2846	No	+	34	F	59	250	H	none detected
2850	No	+	34	M	67	195	H	none detected
2866	No	+	42	F	63	165	H	
2885	No	+	18	F	64	184	H	
2892	No	+	33	F	67	154	H	
2893	No	+	36	F	60	184	H	
								C,D,G,T
2551	Yes	+	46	M	65	210	B	[M,R]
2704	Yes	+	20	M	69	152	H	C,D
2720	Yes	+	55	M	72	166	W	
								C,D,M,T
2847	Yes	+	34	F	67	182	H	[G,R]
2864	Yes	+	55	F	68	174	B	
2868	Yes	+	32	F	67	163	W	
2871	Yes	+	32	F	63	120	H	C,G
2875	Yes	+	53	F	64	155	H	
								C,D,G,T
2879	Yes	+	53	M	72	250	B	[M,R]
2891	Yes	+	39	F	64	185	H	
2886^	Yes	0	19	F	62	115	H	C,D,G,M,R,T
2325	Yes	0	30	F	65	160	H	
2696	Yes	0	22	F	62	98	W	
2869	Yes	0	40	F	65	258	W	
2870	Yes	0	29	F	63	160	H	
2874	Yes	0	21	F	63	100	H	
2876	Yes	0	32	F	64	237	A	C,G
2878	Yes	0	43	M	68	230	H	C,D,G [R,T]
2883	Yes	0	29	F	65	172	W	C,G,T
2888	Yes	0	32	F	60	156	H	
2890	Yes	0	26	F	68	140	H	

*A Asian, B African-American, H Hispanic, W white non-Hispanic.

†By skin-prick test; C cat dander, D dust mite, G grass pollen, M mold, R ragweed, T tree pollen. Blanks indicate data not available. Brackets indicate slight (grade +1) response.

^Second exposure (to CAPS) was terminated before completion at subject's request, because of gastrointestinal symptoms which had been present beforehand but became worse during exposure, probably due to an acute infection. No post-exposure testing was done. Uneventful recovery followed over the next 2 days. This subject's data are not included in statistical analyses.

Exposures

Table 5 compares means and standard deviations of key environmental measurements between control (FA) and exposure (CAPS) studies. Concentrations of pollutant gases were generally low. (Gases were measured in ambient air upstream of the particle concentrator, rather than in the exposure chamber; prior tests had shown little difference between ambient and in-chamber measurements of gases.) Pollutant gas concentrations, temperature in the chamber, and relative humidity in the chamber generally showed similar ranges in control and exposure conditions. One exception was NO_2, which was significantly higher in CAPS than in FA studies (mean difference 9.6 ppb, $P < 0.05$ by paired t test). This is explainable in that occasionally the day of a scheduled study exposure would have low ambient pollution levels, such that the concentrator would be unable to deliver a CAPS concentration near 200 $\mu g/m^3$. In that situation, if a CAPS exposure had been planned, it would be postponed unless no later opportunity was available. If a FA exposure had been planned, it would proceed as intended. As a result, FA studies occurred more often on low-pollution days and averaged lower in ambient PM than CAPS study days. Since ambient NO_2 and ambient PM concentrations tend to track each other day to day, ambient (and chamber) NO_2 also tended to be lower on FA study days. Ambient conditions averaged warmer and less humid on CAPS relative to FA days, but average differences were small in comparison with day-to-day variation.

Table 5. Environmental Measurements (Mean ± SD) in Filtered Air Control Studies vs. Concentrated Fine Particle Exposures

Measure	Filtered Air Controls	CAPS Exposures
Mass concentration, total filter ($\mu g/m^3$) [a]	35 ± 16	187 ± 42
Mass concentration, DataRAM ($\mu g/m^3$)	13 ± 7	288 ± 55
Mass concentration, MOUDI ($\mu g/m^3$) [a]	16 ± 3 [b]	164 ± 39
O_3 (ppb)	23 ± 11	20 ± 11
NO_2 (ppb)	24 ± 14	34 ± 21
SO_2 (ppb) [c]	1.8 ± 1.3	1.6 ± 0.8
CO (ppm)	1.6 ± 1.2	1.6 ± 1.1
Chamber temperature (^oF)	71 ± 2	72 ± 2
Chamber relative humidity (%)	69 ± 11	70 ± 11
Outdoor temperature (^oF)	76 ± 7	78 ± 7
Outdoor relative humidity (%)	44 ± 13	41 ± 7

[a] Total filter and MOUDI statistics are based on a single integrated sample for each 2-hr exposure period. Others are based on time-weighted average over each exposure period.
[b] 2 samples.
[c] 8 samples filtered air, 7 samples CAPS.

Particle mass concentrations as measured by total filter (considered the most accurate overall mass measurement) averaged 187 µg/m^3 in CAPS and 35 µg/m^3 in FA exposures. The CAPS-FA difference for individual subjects averaged 152 µg/m^3, with standard deviation of 40 and range of 67 to 220. Figure 1 shows the distribution of CAPS particle mass versus size range, as determined from MOUDI samples. A large majority of the mass was in the fine size range, with a mode near 1 µm aerodynamic diameter, as expected. Although concentrations varied appreciably between different CAPS exposures, the percentages of mass in each size range were reasonably consistent. The mean mass concentration in CAPS exposures as estimated from MOUDI data was 164 µg/m^3, about 23 µg/m^3 or 12% below the estimate from total filters. A roughly comparable difference was found in two FA studies with MOUDI measurements: they were 16 and 19 µg/m^3 below the concurrent total-filter estimates. These differences might be due to chance or to slight loss of mass in the process of handling the multiple MOUDI filters. Alternatively, the total filters may have collected extra mass in very large subject-generated particles that would have been excluded from the MOUDI.

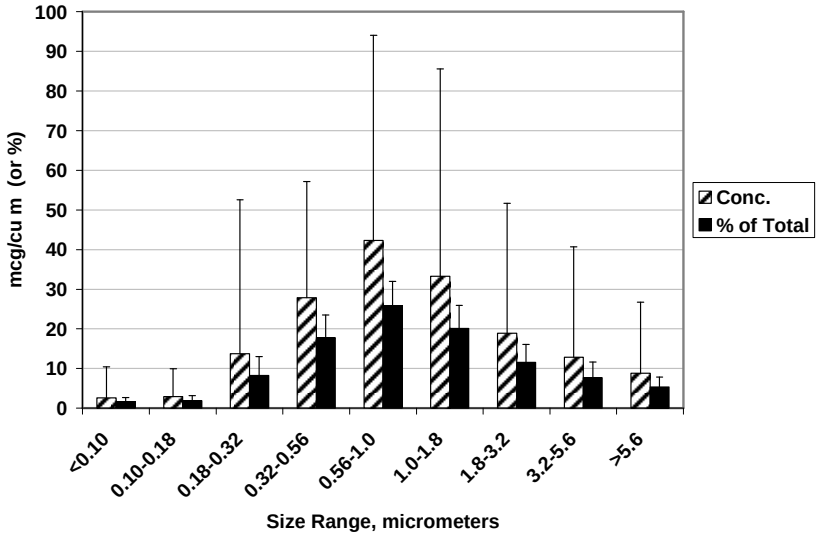

Figure 1. Average particle mass versus size range, as determined by micro-orifice uniform-deposit impactor (MOUDI) sampling, in concentrated ambient fine particle (CAPS) exposures. Bar indicates mean, flag indicates standard deviation. Hatched bars: concentration in µg/m^3; solid bars: concentration as percentage of total for all size ranges.

The appreciable PM concentrations in FA exposures are attributable mostly to particles released from subjects' skin and clothing, with some additional contribution from the exercise cycle, despite efforts to minimize that source. (The chamber interior was carefully pre-cleaned, and subjects wore lint-free scrub suits, head covers, and shoe covers). The range was from 6 to72 µg/m^3, similar to the range in previous studies that employed the same methodology.[94] Periodic tests in which the equipment was operated in FA mode with no subject in the chamber showed nearly zero internal PM mass concentrations, verifying the integrity of the HEPA filter. Also, more detailed monitoring in the above-mentioned earlier studies showed that nitrate, a major component of ambient PM in the Los Angeles area, was extremely low in FA exposures, thus ruling out any appreciable contamination by ambient PM. The highest mass concentrations in FA exposures were found with a few subjects who tended to sweat very freely, and experienced warm and very humid conditions in the chamber toward the end of their exposure periods. For the subject group as a whole, there was only a marginally significant tendency for FA PM concentration to increase with chamber temperature or humidity. It was not practical to perform MOUDI determinations of particle size range routinely in FA exposures, but MOUDI data were collected on two occasions. Those data indicated that, as would be expected, the subject-

generated particles were predominantly coarse particles, above (or at the upper end of) the $PM_{2.5}$ size range. Figure 2 compares the average mass-versus-size distribution of the 2 FA samples against the average from all CAPS exposures.

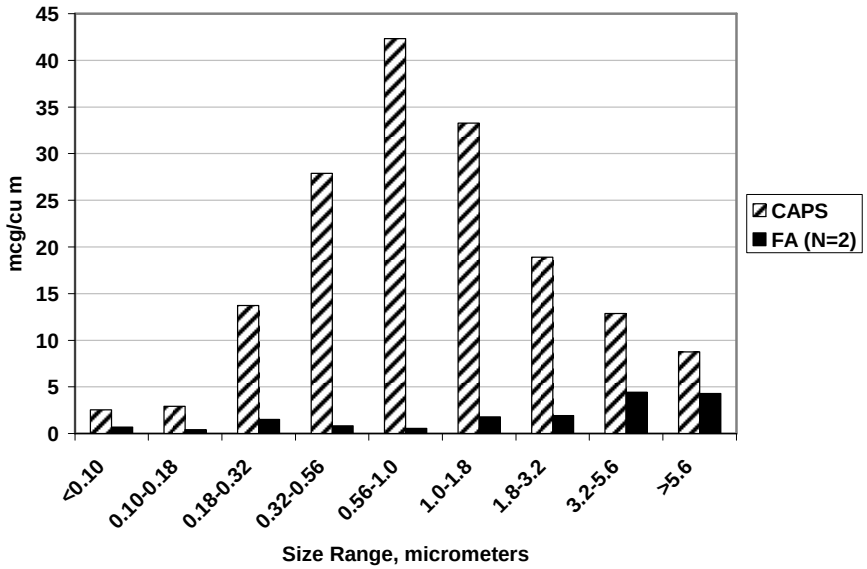

Figure 2. Average particle mass versus size range, as determined by micro-orifice uniform-deposit impactor (MOUDI) sampling, in filtered-air (FA) exposures (2 samples) versus concentrated ambient fine particle (CAPS) exposures (31 samples).

The DataRAM 4 nephelometer (Thermo Scientific, Franklin, MA) had upgraded sample-conditioning capabilities (compared to earlier models used for past CAPS studies in this laboratory), intended to provide more stable and accurate real-time estimates of PM mass concentrations under varying temperature and humidity conditions. Nevertheless, 2-hour average concentrations estimated by the DataRAM were more than 50% above concurrent total-filter measurements, on average (Table 5). The existence of a proportionality factor (DataRAM/gravimetric measurement) >= 1.5 was documented prior to exposure studies. Efforts to define that factor more accurately - multiple test runs of the particle concentrator with no subject in the chamber under various ambient temperature and humidity conditions, and interim data analysis after some subjects' exposures had been completed – met with only limited success. Thus, real-time adjustments of the particle concentrator based on DataRAM readings sometimes resulted in either overshooting or undershooting the intended 2-hour-average exposure concentration of 200 $\mu g/m^3$. Expressed as CAPS-FA difference, the highest 10% of concentrations exceeded the target by 2% (204 $\mu g/m^3$) to 10% (220 $\mu g/m^3$). The median concentration expressed as CAPS-FA difference was 164 and the interquartile range was 120-177 $\mu g/m^3$. The lowest 10% of concentrations fell below 100 $\mu g/m^3$, i.e. below 50% of the target. These low values resulted not from DataRAM uncertainty, but from ambient PM concentrations too low for the concentrator to reach its target. Sometimes that reflected unusually clean weather conditions on a day when a CAPS study was scheduled and could not be rescheduled. More often, ambient PM levels were sufficiently high at the start of the exposure, but fell markedly toward the end of the exposure, due to a rise in wind speed. Figure 3 illustrates the imprecise relationship between DataRAM and total filter concentration measurements (r = 0.52). By contrast, Figure 4 illustrates the usually excellent agreement between two total filter samples collected from different chamber sampling ports (r = 0.89 including one outlying data point, r = 0.98 excluding that point). Figure 5 shows the relationship between total filter and MOUDI data (r = 0.72).

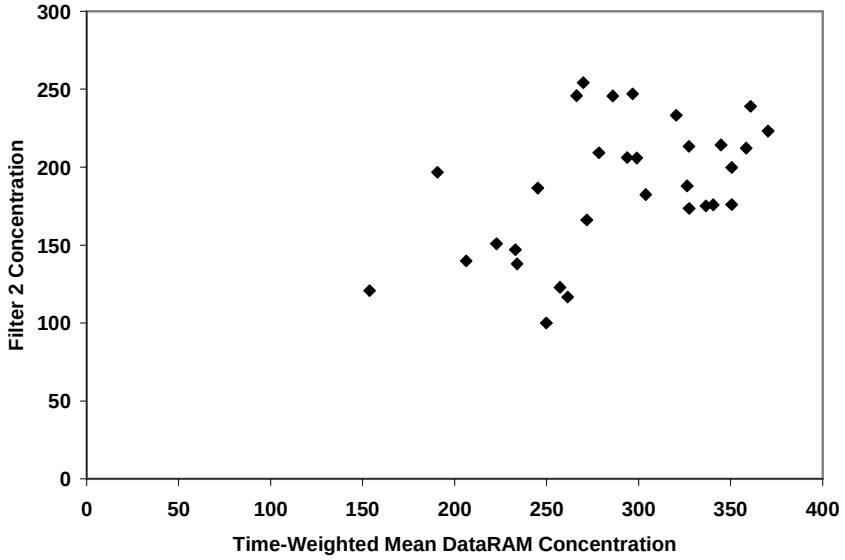

Figure 3. Exposure concentration measured by total filter, as a function of corresponding time-weighted-average DataRAM nephelometer measurement. Agreement was poor (r = 0.51).

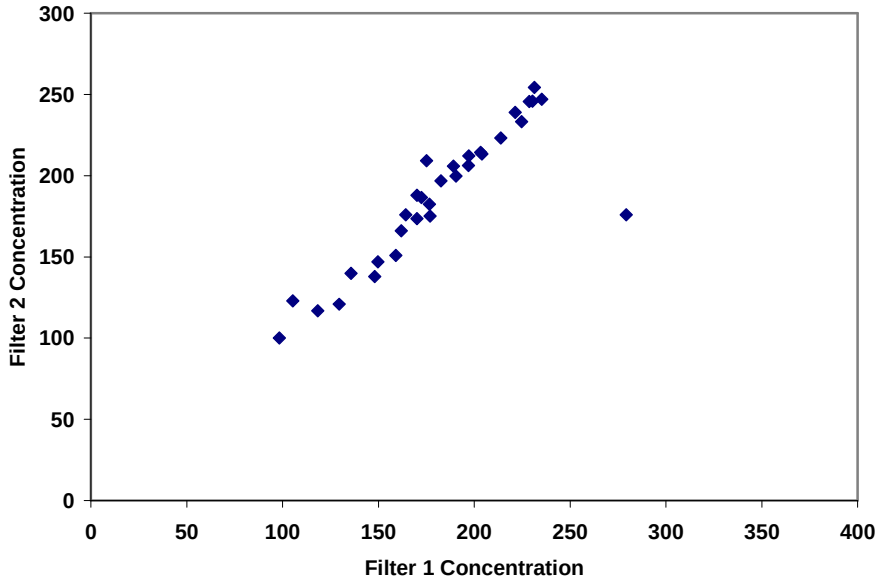

Figure 4. Exposure concentration measured by total filter, versus that measured concurrently by a second total filter sample. Measurements disagreed appreciably in only one exposure study. There the MOUDI concentration corroborated the filter-2 measurement, suggesting that the high value from filter 1 was spurious.

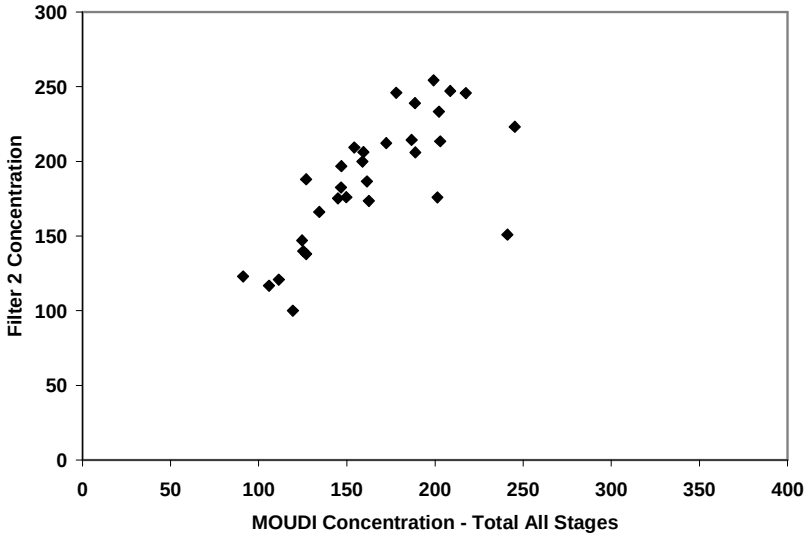

Figure 5. PM mass concentration measured by total filter sample in CAPS exposures, vs. concurrent mass concentration measured by MOUDI (r = 0.72).

Thus, concentrated fine particle exposure concentrations varied appreciably from subject to subject, as was true in previous concentrator studies. Causes of this variation include imprecision of the real-time particle monitoring instrument that was used to determine what fraction of the concentrator's output should be directed to the exposure chamber, and variability of outdoor ambient fine particle concentrations during exposure periods. At times the outdoor concentrations were so low that the concentrator's output fell appreciably below the target level, even with 100% of the output delivered to the exposure chamber. Also, despite the fact that ambient particulate pollution was rigorously excluded from filtered-air exposures, their particle mass concentrations were nontrivial, and varied substantially between subjects. This was due to the "personal cloud" phenomenon enhanced by crank cycle exercise, in a confined space with relatively slow air exchange. In light of this variability, health endpoints are statistically analyzed in dose-response fashion (with exposure PM concentration difference, CAPS-FA, as a continuous predictor variable) as well as in the usual control-versus-exposure fashion (with CAPS vs. FA exposure condition as a fixed factor).

Physiologic and Symptom Responses

Table 6 shows mean pre-exposure physiologic measurements and symptom scores for the 3 groups defined by asthma/GSTM1 status. Group differences were not significant, although the differences in FEV_1/FVC ratio approached significance ($P < 0.1$), and mean FeNO was higher in asthmatic than in healthy subjects, as expected.

Table 6. Pre Exposure Physiologic and Symptom Measurements: Mean and (Standard Deviation) by Group [a]

	Healthy	Asthma GSTM1 +	Asthma GSTM1 null
Symptom Score [b]	1.1 (2.0)	2.7 (3.3)	1.6 (2.4)
FVC (ml)	4038 (850)	3786 (550)	4044 (796)
FEV_1 (ml)	3298 (686)	3002 (481)	3050 (622)
FEV_1/FVC (%)	81.9 (4.4)	79.5 (7.9)	75.6 (6.4)
BP systolic (mmHg)	115 (12)	109 (10)	111 (12)
BP diastolic (mmHg)	74 (11)	74 (9)	73 (12)
SaO_2 (%)	98.5 (0.9)	98.2 (2.0)	98.4 (1.3)
FeNO (ppb)	26 (13)	50 (53)	42 (30)
FeCO (ppm)	1.3 (0.9)	1.3 (1.2)	1.0 (0.8)

[a] FVC: forced vital capacity. FEV_1: forced expired volume in one second. BP: blood pressure. SaO_2: arterial oxygen saturation as estimated by fingertip pulse oximetry. FeNO: exhaled concentration of nitric oxide. FeCO: exhaled concentration of carbon monoxide.

[b] Sum for 17 different symptoms recorded on questionnaires; each scored 0 = not present, 1 = mild, 2 = moderate, 3 = severe, 4 = incapacitating

Table 7 summarizes results from mixed-model analyses of each variable using SAS procedure MIXED (SAS Statistical Software, Cary, NC) to estimate effects of grouping factors and atmosphere (CAPS vs. FA) on the response to exposure, i.e. the change (Δ) measured immediately after exposure ("post") or the next morning ("day 2"), relative to pre-exposure. Subject was treated as a random factor in these analyses; group and atmosphere were treated as fixed factors. Group differences were tested by estimating separate effects of asthma status (healthy vs. asthmatic) and GSTM1 status (null vs. positive), and also by testing differences across the 3 distinct groups (healthy, asthmatic GSTM1-positive, asthmatic GSTM1-null). Analyses were performed with both group and atmosphere, group only, and atmosphere only in the model. Table 8 presents key results from models that simultaneously tested effects of asthma status, GSTM1 status, and atmosphere; other models gave mostly similar results. Overall responses to the experimental protocol, as well as differences related to group or to exposure atmosphere, were mostly small and non-significant. Exceptions are discussed below.

Table 7. Summary of Mixed-Model Analyses of Physiology and Symptom Data [a]

Measure of Response	Significant ($P < 0.05$) Results
Symptom score during exp.[b]	increase from pre-exposure, larger in GSTM1-null
Symptom score after exp.[c]	increase from pre-exposure in GSTM1-null only
ΔFVC post - pre or day 2 - pre	(none)
ΔFEV_1 post – pre or day 2 - pre	(none)
ΔBP systolic post – pre (mmHg)	decrease from pre-exposure, less in GSTM1-null
ΔBP systolic day 2 – pre (mmHg)	(none)
ΔBP diastolic post–pre or d2-pre	(none)
ΔSaO_2 post – pre (%)	(none)
ΔFeNO [d] post – pre (ppb)	increase after CAPS relative to FA
ΔFeNO [d] day 2 – pre (ppb)	(none)
ΔFeCO [d] post–pre or day 2 – pre	(none)

[a] See Table 6 for explanation of abbreviations; see text and Table 9 for statistical details.
[b] Change in symptom score during exposure relative to pre-exposure, averaged over 8 measurements at 15-min intervals; see table 6 for scoring method.
[c] Change in symptom score after exposure relative to pre-exposure, averaged over immediate post, 1 hr post, 2 hr post, and day 2 measurements.
[d] Adjusted for concurrent change in ambient NO or CO concentration. Ambient NO effect was significantly positive ($P < 0.05$); ambient CO effect was non-significant.

Table 8. Mixed-Model Analyses of Physiology and Symptom Data: Estimates and (Standard Errors) from Models with Asthma, GSTM1, and Atmosphere Main Effects, No Interaction Terms [a]

Variable	Intercept [b]	Asthma status [c]	GSTM1 status [d]	Atmo-sphere [e]
Symptom score during exp.	+2.4 (1.4)	-0.3 (1.9)	+4.5 (1.9)*	+0.02 (0.9)
Symptom score after exp.	-0.1 (0.7)	-0.3 (0.9)	+2.2 (0.9)*	+0.7 (0.5)
ΔFVC post - pre (ml)	-26 (56)	+33 (76)	-17 (76)	-46 (37)
ΔFVC day 2 – pre (ml)	+5 (60)	-29 (75)	-9 (75)	-70 (51)
ΔFEV_1 post – pre (ml)	+50 (40)	-22 (47)	-10 (47)	-31 (27)
ΔFEV_1 day 2 – pre (ml)	+50 (44)	-60 (53)	-9 (53)	-50 (41)
ΔBP systolic post – pre (mmHg)	-10 (2.2)*	+3.9 (2.8)	+6.2 (2.8)*	+0.4 (2.0)
ΔBP systolic day 2 – pre (mmHg)	-5.5 (2.7)*	+2.7 (3.3)	+3.5 (3.3)	+4.1 (2.6)
ΔBP diastolic post – pre (mmHg)	-2.0 (2.6)	+4.6 (3.6)	-2.4 (3.6)	+0.5 (1.7)
ΔBP diastolic day 2–pre (mmHg)	-0.1 (2.6)	-0.1 (3.4)	-1.1 (3.4)	+0.1 (2.1)
ΔSaO_2 post – pre (%)	-0.6 (0.5)	+0.4 (0.6)	+0.2 (0.6)	-0.02 (0.4)
ΔSaO_2 day 2 – pre (%)	-0.1 (0.3)	+0.1 (0.4)	+0.5 (0.4)	-0.2 (0.3)
ΔFeNO post – pre (ppb)	+2.6 (3.4)	-0.6 (4.3)	-1.7 (4.3)	-6.0 (2.1)*
ΔFeNO day 2 – pre (ppb)	-2.0 (2.5)	+1.1 (3.1)	+1.3 (3.2)	-1.3 (2.6)
ΔFeCO post – pre (ppm)	-0.1 (0.3)	-0.3 (0.4)	-0.1 (0.4)	-0.2 (0.3)
ΔFeCO day 2 – pre (ppm)	-0.4 (0.4)	+0.9 (0.5)	+0.5 (0.5)	+0.3 (0.4)

[a] Statistically significant ($P < 0.05$) effects are indicated by *; see text for further discussion. See Tables 6 and 7 for explanation of variables.

[b] Intercept represents estimate for the "reference" condition: GSTM1-positive asthmatic subject in CAPS exposure.

[c] Estimate for healthy subject, relative to asthmatic.

[d] Estimate for GSTM1-null subject, relative to GSTM1-positive subject.

[e] Estimate for FA exposure, relative to CAPS.

Systolic blood pressure showed a significant ($P < 0.001$) overall decrease post- relative to pre-exposure, essentially the same with CAPS and FA. This may be attributable to some combination of circadian variation and response to the stresses inherent in the experimental protocol. Possibly, anticipation of exposure stresses (confinement, exercise) tended to raise blood pressure before exposure, while relief of those stresses tended to lower it after exposure. In terms of group means, the decrease was largest in GSTM1-positive asthmatics, and smallest in GSTM1-null asthmatics (Figure 6). Group differences were non-significant in most analytical models ($P \sim 0.1$). However, in a model with atmosphere, asthma status, and GSTM1 status as predictors with no interaction terms, GSTM1 status was significant ($P = 0.04$), with a point estimate of 6 mmHg smaller loss in systolic pressure for GSTM1-null compared to GSTM1-positive subjects. In a similar model, GSTM1-null asthmatics showed significantly ($P = 0.02$) greater symptom increases during exposure than GSTM1-positive subjects: estimated mean score increases were 6.8 and 2.3 points respectively (both significantly different from zero). Symptom scores after exposure were not significantly different from pre-exposure in GSTM1-positive subjects, but remained elevated in GSTM1-null asthmatics (estimated increase 2.5 points). Asthma status had no significant effect on any measure of response. None of these results differed significantly between CAPS and FA exposures.

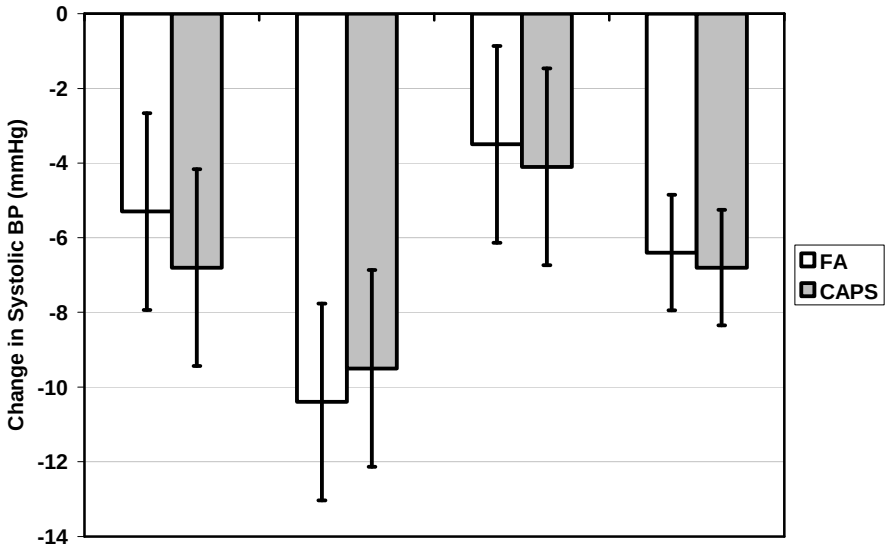

Figure 6. Mean change in systolic blood pressure pre- to post-exposure, FA vs. CAPS, for each group and for all subjects pooled. Column = mean, flag = standard error. H= healthy, A+ = asthma GSTM1-positive, Ao = asthma GSTM-1 null.

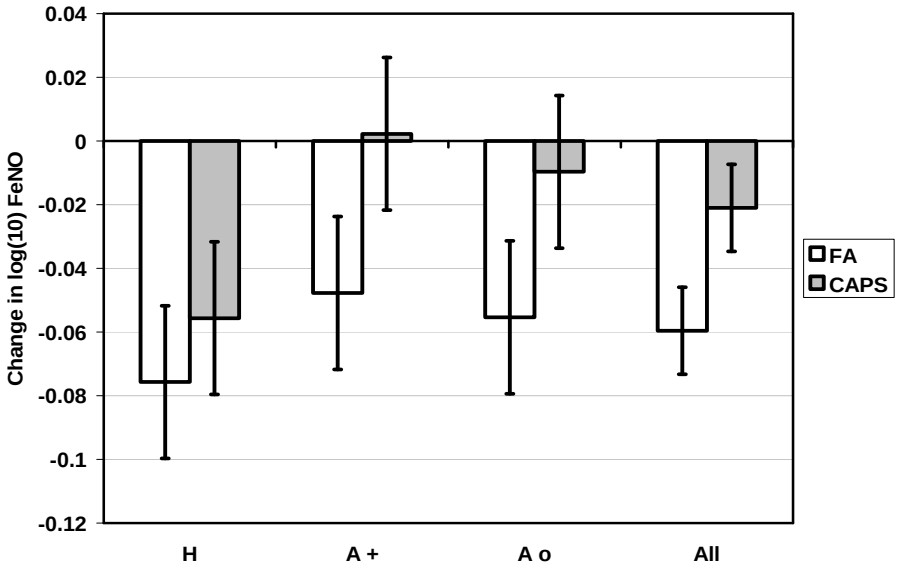

Figure 7. Mean change in log-transformed FeNO pre- to post-exposure, FA vs. CAPS, for each group and for all subjects pooled. Column = mean, flag = standard error. H= healthy, A+ = asthma GSTM1-positive, Ao = asthma GSTM-1 null.

As Tables 7 and 8 indicate, exhaled nitric oxide (FeNO) was the only variable found to respond more unfavorably to CAPS than to FA exposure. The estimated mean increase post-exposure with CAPS relative to FA was 6 ppb, after adjustment for effects of asthma and GSTM1 status (non-significant) and for the significant effect of ambient NO concentration at the time of FeNO measurement. The latter effect may be an artifact of ambient air admixture in exhaled NO samples, since the testing system (Sievers Model 280i, GE Analytical Instruments, Boulder, CO) was not equipped to remove ambient NO from inhaled air. Alternatively, ambient NO or accompanying pollutants may acutely affect subjects' airways in a manner that increases NO excretion. The CAPS effect on FeNO did not vary significantly according to asthma or GSTM1 status, although the mean CAPS-FA difference was larger in asthmatic than in healthy subjects (Figure 7). The effect remained significant in analyses with NO concentrations log-transformed to normalize data distributions, with or without adjustment for ambient NO, asthma, and GSTM1 effects. It was not explainable by pre-exposure differences in FeNO or ambient NO between CAPS and FA exposures, which were small and non-significant.

For the alternative "dose-response" analyses of exposure effects, individuals' net changes in response measures – spirometry, blood pressure, FeNO – were calculated as the change pre- to immediately post-CAPS exposure, minus the corresponding change with FA. For symptom score, the average change during exposure was used rather than the pre-to-post change. Correlations were then computed between these net changes and individual CAPS-FA exposure differences, for all 30 subjects. Table 9 shows results. No response measure was significantly related to PM exposure concentration as measured by gravimetric (filter) sampling or by DataRAM nephelometer. Symptom score change was significantly related to exposure NO_2 concentration, but in the "wrong" direction: higher NO_2 during exposure was associated with less increase in symptoms. Linear regression analysis yielded an exposure-response slope estimate of -0.16 points change in symptom score per 1 ppb increase in average NO_2 concentration during exposure (standard error 0.04, P = 0.0003). A similar analysis with PM concentration (by filter) as the exposure variable estimated a non-significant slope of -0.031 points change in symptom score per 1 $\mu g/m^3$ increase in concentration.

Table 9. Correlations of Response Measures (Net Change during CAPS Exposure Relative to FA) with Exposure Measures (Difference CAPS-FA) [a]

	FVC	FEV_1	BP systolic	BP diastolic	SaO_2	Symptom Score	FeNO
Concentration (filter sample)	-0.01	+0.04	-0.28	+0.19	+0.25	-0.20	-0.15
Concentration (DataRAM)	+0.07	+0.19	-0.11	-0.06	-0.00	-0.29	+0.11
NO_2	+0.15	+0.34	-0.04	-0.29	+0.33	**-0.63** (P < .001)	-0.12
Chamber Temperature	-0.02	-0.17	-0.02	+0.14	+0.05	+0.13	+0.14

[a] Correlations were non-significant (P > 0.05) except for symptom score vs. NO_2.

Immunologic Data

Each cytologic and immunologic variable was subjected to analysis of variance with repeated measures on subjects, using SAS procedure MIXED (SAS Statistical Software, Cary, NC) to estimate effects of susceptibility group (healthy vs. asthmatic GSTM1-positive vs. asthmatic GSTM1-null), atmosphere (CAPS vs. FA), and time (pre-exposure baseline [B] vs. 2 hr post-exposure [P2] vs. 22 hr post-exposure [P22]). Sputum data were given the most attention, being most directly relevant to the hypothesis of increased lower-airway response in GSTM1-null asthmatics. As previously discussed, pre-exposure baseline sputum induction was not feasible due to potential effects on exposure responses, so a baseline measurement was taken during a third laboratory visit with no exposure study. Subject was treated as a random factor in these analyses; group, atmosphere, and time were treated as fixed factors. The MIXED procedure was used because it could handle unbalanced designs easily. Unbalance occurred for some variables because a few data were missing due to unsuccessful sample collection or problems in sample handling.

Selected variables were analyzed in more detail, by "advanced" statistical models testing effects of period (first vs. second vs. third laboratory visit) and/or sequence (a classification of subjects by the particular order in which they experienced the different experimental conditions), in addition to the effects tested in the aforementioned "conventional" statistical models. This would test for effects due to the passage of time, the so called period effect, on the observed responses to CAPs, but not for confounding by carryover effects from a previous exposure, as these were expected to be minimal by design. Although period effects were significant in some instances, conclusions from "conventional" and "advanced" models concerning significance of CAPs effects were usually the same. Thus, "conventional" results are reported here, unless noted otherwise.

To assess the need for data transformation to normalize distributions, overall data distributions were examined for all variables, and distributions of residuals from "conventional" analytical models, broken down by levels of the repeated-measures factor, were examined for selected variables. For variables that were quantifiable in all cases, distributions were typically non-normal on the original scale but close to normal after log transformation. For samples with undetectable concentrations of some species, the value of each affected variable was estimated as one-half the lowest quantifiable measurement, to allow log-transformation. After transformation, distributions of those variables were improved but still non-normal. In general, data were analyzed both on the original scale and after log-transformation. Usually the statistical conclusions were similar either way.

Sputum

Sputum inductions could not be performed both pre- and post-exposure like other test procedures, because of expected carryover effects and excessive subject discomfort. Accordingly, a "baseline" sputum sample was taken on a day with no other intervention, usually at least 6 days before/after the closest exposure study (4 days after in one case, 3 days before in one other case). Post-exposure sputum inductions took place on the mornings following CAPs and filtered-air exposures (at time point P22). Data capture was 95% for differential cell counts, and 97-100% for other variables. Table 10 compares results of sputum analyses between baseline, post-FA, and post-CAPs conditions, as estimated from a "conventional" model testing the main and interactive effects of condition and susceptibility group. The tabulated statistics represent untransformed data. If conclusions were appreciably different with log-transformed data, or with comparisons of FA and CAPS only, or with "advanced" models, the differences are described in notes at the end of the table. Sputum eosinophils, IgE, interferon-gamma, GMCSF, and TNF-alpha were not statistically analyzed in this manner because they were not detected in a majority of samples.

Table 10. Results of Mixed-Model Analyses on Sputum Variables Including Baseline, Post-Filtered-Air, and Post-CAPs Measurements. (Subject as random factor, susceptibility group and condition as fixed factors; analytical model tests main and interactive effects of fixed factors.)

Total Cell Count (millions/ml)

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	1.11	0.28	0.93	0.2	0.47
Asth Null	FA	0.75	0.27			
Asth Null	base	0.94	0.27		[Note A]	
Asth Pos	CAPS	1.33	0.27			
Asth Pos	FA	1.03	0.27			
Asth Pos	base	0.81	0.27			
Heal Pos	CAPS	1.00	0.27			
Heal Pos	FA	0.98	0.27			
Heal Pos	base	1.03	0.27			

Percent Monocytes

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	48.00	6.34	0.022	0.093	0.93
Asth Null	FA	54.80	6.34			
Asth Null	base	55.90	6.34		[Note B]	
Asth Pos	CAPS	29.80	6.34			
Asth Pos	FA	30.08	6.57			
Asth Pos	base	35.08	6.57			
Heal Pos	CAPS	41.70	6.54			
Heal Pos	FA	48.20	6.34			
Heal Pos	base	51.99	6.54			

Percent Lymphocytes

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	0.80	0.34	0.53	0.035	0.49
Asth Null	FA	1.60	0.34			
Asth Null	base	1.70	0.34		[Note C]	
Asth Pos	CAPS	1.60	0.34			
Asth Pos	FA	1.34	0.35			
Asth Pos	base	2.23	0.35			
Heal Pos	CAPS	1.32	0.35			
Heal Pos	FA	1.30	0.34			
Heal Pos	base	1.78	0.35			

Table 10 (continued). Results of Mixed-Model Analyses on Sputum Variables Including Baseline, Post-Filtered-Air, and Post-CAPs Measurements. (Subject as random factor, susceptibility group and condition as fixed factors, analytical model tests main and interactive effects of fixed factors.)

Percent PMNs

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	51.10	6.39	0.038	0.072	0.97
Asth Null	FA	46.40	6.39			
Asth Null	base	42.40	6.39			
Asth Pos	CAPS	68.20	6.39			
Asth Pos	FA	67.69	6.63			
Asth Pos	base	62.03	6.63			
Heal Pos	CAPS	56.72	6.60			
Heal Pos	FA	50.30	6.39			
Heal Pos	base	46.05	6.60			

IgA (ng/ml)

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	4.82	5.31	0.003	0.15	0.12
Asth Null	FA	6.19	5.31			
Asth Null	base	5.59	5.31			
Asth Pos	CAPS	2.62	5.31			
Asth Pos	FA	2.25	5.31			
Asth Pos	base	2.71	5.31			
Heal Pos	CAPS	16.28	5.31			
Heal Pos	FA	10.24	5.31			
Heal Pos	base	32.44	5.31			

IgG (ng/ml)

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	20.13	9.26	0.29	0.17	0.32
Asth Null	FA	27.43	9.26			
Asth Null	base	24.19	9.26			
Asth Pos	CAPS	18.27	9.26			
Asth Pos	FA	13.23	9.26			
Asth Pos	base	21.38	9.26			
Heal Pos	CAPS	28.69	9.26			
Heal Pos	FA	22.59	9.26			
Heal Pos	base	49.27	9.26			

Table 10 (continued). Results of Mixed-Model Analyses on Sputum Variables Including Baseline, Post-Filtered-Air, and Post-CAPs Measurements. (Subject as random factor, susceptibility group and condition as fixed factors, analytical model tests main and interactive effects of fixed factors.)

IgM (ng/ml)						
Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	21.98	3.99	0.23	0.84	0.48
Asth Null	FA	27.24	3.99			
Asth Null	base	23.82	3.99			
Asth Pos	CAPS	22.41	3.99			
Asth Pos	FA	20.51	3.99			
Asth Pos	base	21.99	3.99			
Heal Pos	CAPS	29.97	3.99			
Heal Pos	FA	25.95	3.99			
Heal Pos	base	31.88	3.99			
IgG4 (ng/ml)						
Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	2.10	6.19	0.11	0.47	0.65
Asth Null	FA	5.04	6.19			
Asth Null	base	2.58	6.19			
Asth Pos	CAPS	2.17	6.19			
Asth Pos	FA	1.21	6.19			
Asth Pos	base	2.91	6.19			
Heal Pos	CAPS	10.42	6.19			
Heal Pos	FA	16.96	6.19			
Heal Pos	base	21.65	6.19			
IL4 (pg/ml)						
Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	22.58	18.45	0.037	0.0002	0.0332
Asth Null	FA	27.01	18.45			
Asth Null	base	102.18	18.45		[Note D]	
Asth Pos	CAPS	57.38	19.06			
Asth Pos	FA	44.05	18.45			
Asth Pos	base	84.18	18.45			
Heal Pos	CAPS	5.32	18.45			
Heal Pos	FA	4.15	18.45			
Heal Pos	base	12.03	18.45			

Table 10 (continued). Results of Mixed-Model Analyses on Sputum Variables Including Baseline, Post-Filtered-Air, and Post-CAPs Measurements. (Subject as random factor, susceptibility group and condition as fixed factors, analytical model tests main and interactive effects of fixed factors.)

IL5 (pg/ml)

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	8.09	8.69	0.38	0.33	0.92
Asth Null	FA	11.72	8.69			
Asth Null	base	15.66	8.69			
Asth Pos	CAPS	9.35	8.69			
Asth Pos	FA	7.90	8.69			
Asth Pos	base	20.98	8.69			
Heal Pos	CAPS	24.37	8.69			
Heal Pos	FA	20.39	8.69			
Heal Pos	base	26.12	8.69			

IL8 (pg/ml)

Group	Cond	Estimate	SE	P(Group)	P(Cond)	P(Inter)
Asth Null	CAPS	65.90	31.09	0.15	0.21	0.75
Asth Null	FA	70.28	31.09			
Asth Null	base	87.59	31.09			
Asth Pos	CAPS	45.83	32.42			
Asth Pos	FA	44.20	31.09			
Asth Pos	base	54.64	31.09			
Heal Pos	CAPS	90.88	32.53			
Heal Pos	FA	96.09	31.09			
Heal Pos	base	159.12	32.53			

A. For log-transformed total cell count, using conventional analytical model with baseline data excluded, CAPS-FA difference estimate is 27% (P = 0.052) averaged across susceptibility groups, which are not significantly different. Using advanced analytical model adjusting for the effect of period (i.e. 1st vs. 2nd vs. 3rd lab visit after screening), corresponding CAPS-FA difference estimate is 45% (P = 0.008). See Figure 10 for group estimates from advanced model, Figure 11 for plots of individual data.

B. For log-transformed % monocytes, both group and condition are significant, P = 0.045, 0.012 respectively; CAPS-FA difference is nonsignificant with baseline data excluded. See Figure 8.

C. For log-transformed % lymphocytes, CAPS-FA difference is nonsignificant with baseline data excluded. See Figure 8.

D. For log-transformed IL4, with all data included the difference between susceptibility groups is nonsignificant, but the condition difference is significant: estimates for FA and CAPS (as % of baseline) are 13% and 11% respectively. With baseline data excluded, CAPS-FA difference and group difference are nonsignificant. See Figure 9.

Sputum total cell counts, IgG, IgM, IgG4, IL-5, and IL-8 showed no statistically significant variation among the 3 conditions (baseline, post-FA, post-CAPS) or the 3 susceptibility groups. IgA, percent monocytes, and percent polymorphonuclear leukocytes (PMNs) showed significant differences by group, but not by condition, with this analytical model. That is, underlying asthma/genotype status appeared to influence those 3 variables, but experimental interventions did not. GSTM1-positive asthmatics were low in monocytes and high in PMNs, relative to healthy subjects or to GSTM1-null asthmatics. GSTM1-positive asthmatics were also lowest in IgA; null asthmatics were intermediate and healthy subjects highest.

The percentage of lymphocytes varied significantly by condition, but not by susceptibility group, averaging higher in baseline sputum inductions than after either FA or CAPs exposures. Also, monocytes averaged higher and PMNs lower in baseline sputum inductions, to a degree approaching significance ($0.10 > P > 0.05$). Thus, there may have been some lower-airway response to the experimental exposure experience, with or without CAPs. (The CAPs-FA difference was tested specifically in separate analyses; see below.) Figure 8 shows the estimated means for each of these cell types, by group and condition.

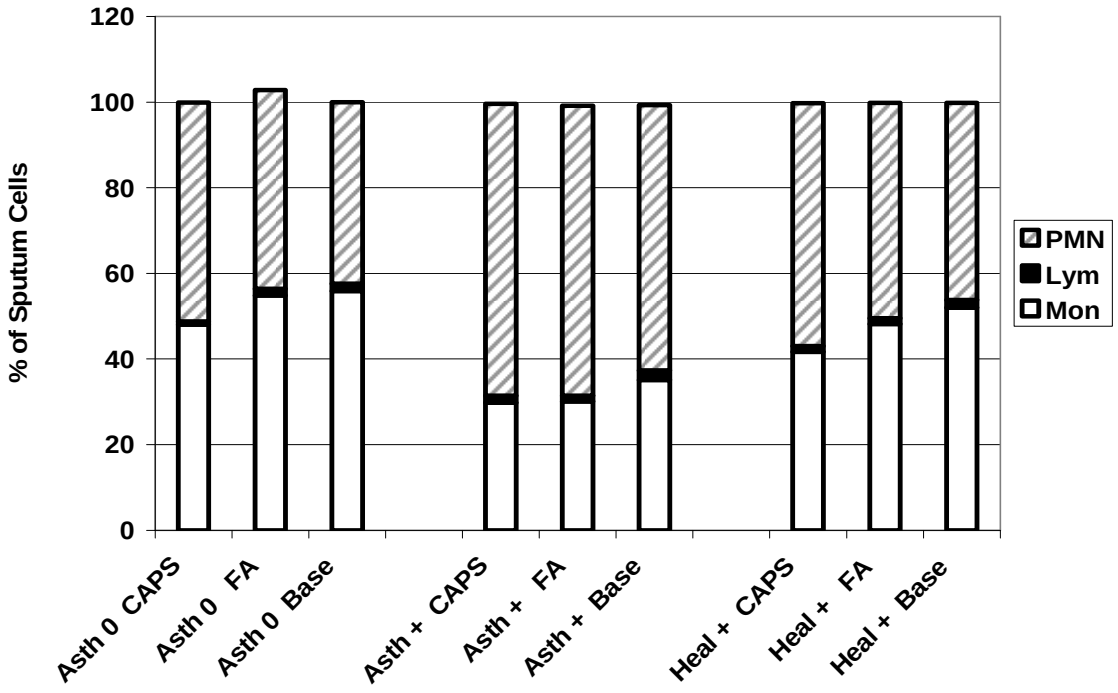

Figure 8. Estimated Mean Percentage of Monocytes, Lymphocytes, and Polymorphonuclear Leukocytes (PMNs) in Induced Sputum, by Susceptibility Group and Induction Condition. (Totals do not add exactly to 100%, because of uncertainties in estimates by mixed-model analyses with occasional missing data, and because eosinophils are not included.)

Interleukin-4 was the only sputum variable to show both main and interactive effects of susceptibility group and condition with untransformed data (Figure 9).

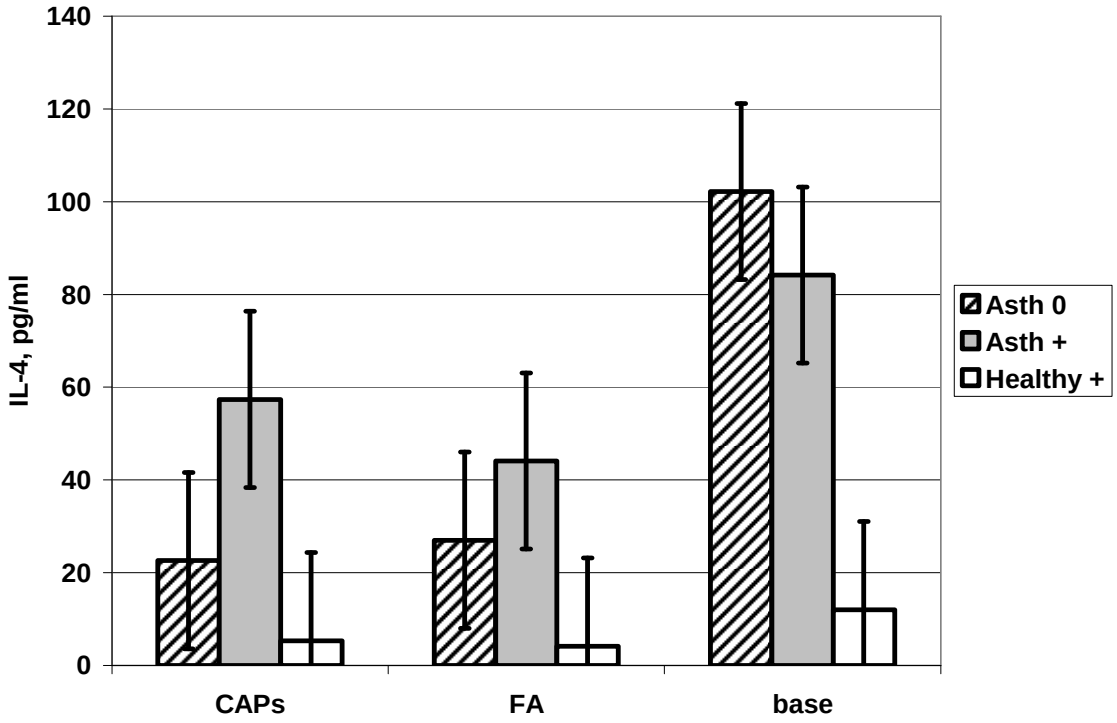

Figure 9. Estimated Mean Concentration of Interleukin-4 in Induced Sputum, by Susceptibility Group and Induction Condition. (Column indicates estimated mean, flag indicates estimated standard error.)

These results must be interpreted with caution, since 6 of 30 subjects never had IL4 detected in their sputum samples, and 16 others had undetectable concentrations in at least one sample. Both asthmatic groups had consistently higher mean IL4 than healthy subjects, and all groups showed decreases in mean IL4 after either exposure, relative to baseline sputum induction. The proportionate decrease differed between groups, being largest in GSTM1-null asthmatics and smallest in GSTM1-positive asthmatics. For log-transformed IL4, the condition difference was still significant – both post-FA and post-CAPs measurements were appreciably lower than baseline – but group differences were nonsignificant.

A separate set of analyses addressed differences in sputum variables between CAPS and FA (ignoring baseline data), testing whether the overall CAPS-FA difference departed from zero, and whether susceptibility groups differed. There was no significance for differential cell counts, IgM, IgG4, IL4, or IL8. Total cell count showed no significance in this analysis, but did show a CAPS-FA difference with an "advanced" statistical model (see below). The estimated mean difference in IgA and (standard error) was +6.0 (2.2) ng/ml in healthy subjects (different from zero, P =0.01), +0.4 (2.2) in GSTM1-positive asthmatics, and -1.4 (2.2) in GSTM1-null asthmatics. The overall between-group variation approached significance (P=0.06). Other

variables were not analyzed in this manner because too many samples had undetectable concentrations.

When the CAPs-vs.-FA analyses were repeated with an "advanced" model including the effect of period, the significance pattern changed for one variable, total cell count. The atmosphere effect became significant (P =0.02) and the period-by-atmosphere interaction approached significance (P = 0.07). Total cell counts after CAPs exposure for subjects exposed in period 1 (first lab visit) tended to be lower than counts after FA exposure for subjects exposed in period 1; but for exposures in periods 2 or 3, counts tended to be higher after CAPS than after FA. The explanation for this pattern (assuming it is not a chance "significant" outcome among many statistical tests) is not obvious. Different results might perhaps be expected from subjects inexperienced and experienced in sputum induction. However, the majority of subjects were already experienced at period 1, having performed sputum inductions in a previous diesel exhaust study. Figure 10 illustrates results from this model. Figure 11 shows plots of sputum total cell counts for each individual and condition: 12 subjects were highest after CAPs, 10 after FA, and 8 at baseline (not significant by rank-sum analysis ignoring sequence).

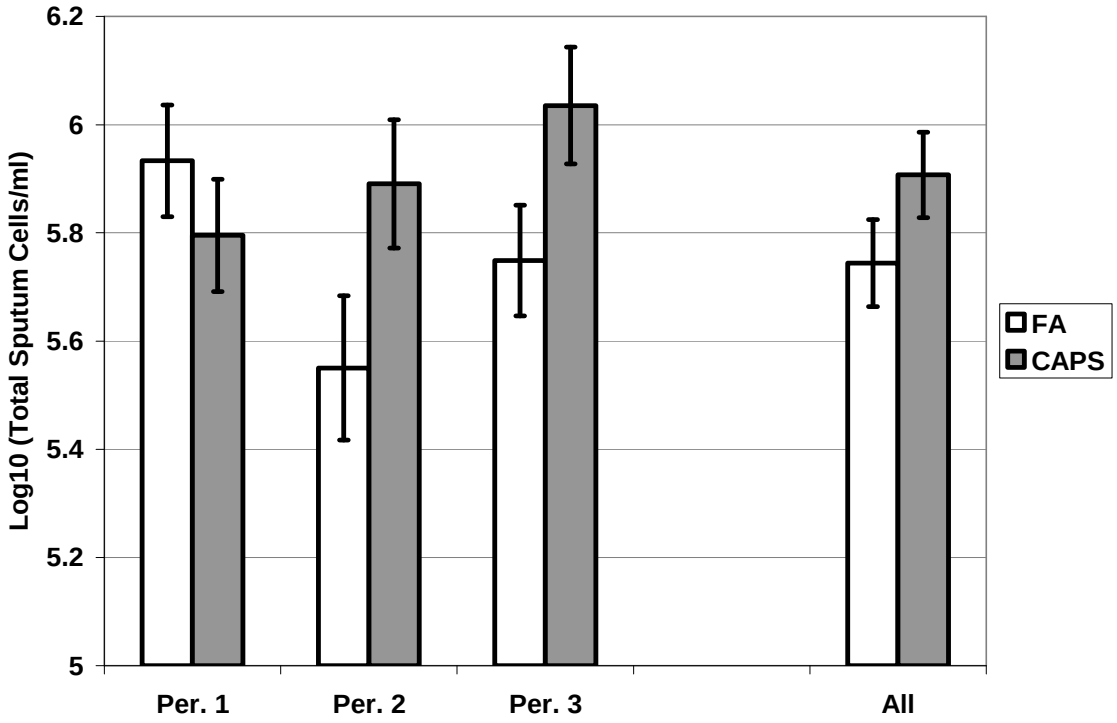

Figure 10. Log-Transformed Total Sputum Cell Count by Exposure Atmosphere, for All Subjects and Separately by Period (i.e. 1st, 2nd, or 3rd Lab Visit for Sputum Induction). (Column indicates estimated mean, flag indicates estimated standard error.)

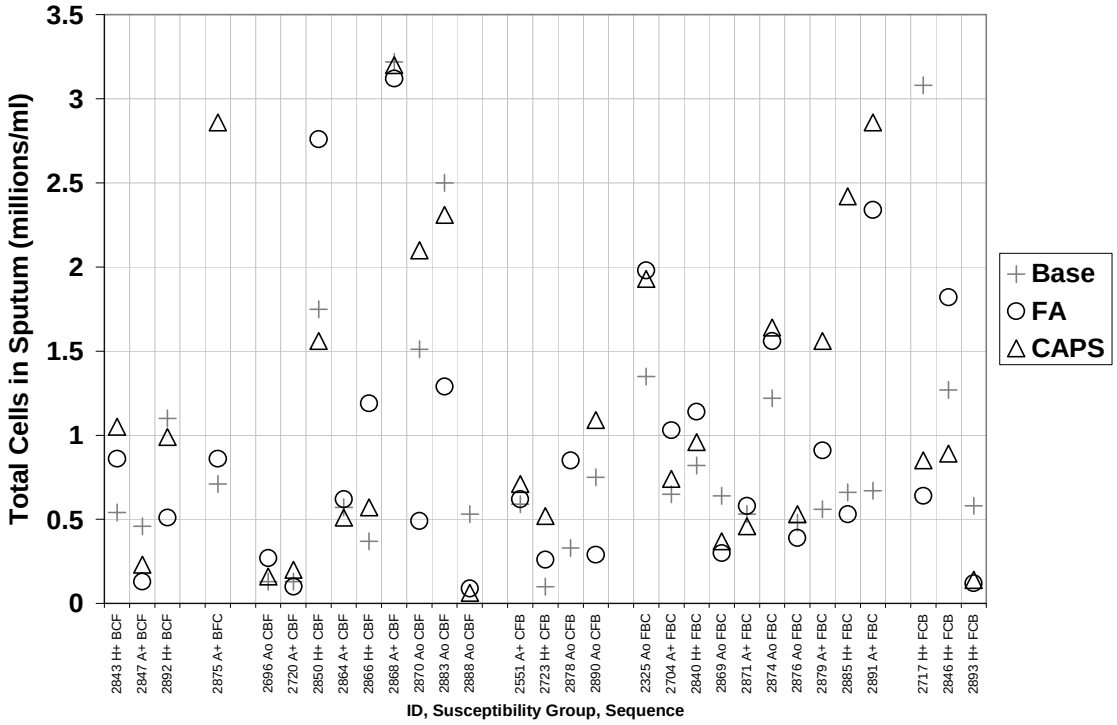

Figure 11. Individual Data: Total Cell Counts from Baseline, Post-FA, and Post-CAPS Sputum Inductions. (Subjects with the same sequence of experimental conditions are grouped together.)

For sputum variables with many measurements below detection limits, results were categorized as 0 = nondetectable or 1 = detectable (ignoring quantity). The proportions of detectable values were then compared between FA and CAPS exposures, for all subjects pooled (Table 11) and for the separate susceptibility groups (data not shown). All the differences were nonsignificant.

Table 11. Percentage of Subjects with Detectable Concentrations of Usually Nondetectable Biomarkers in Sputum: Comparison between Filtered Air and CAPS*

	% after FA	% after CAPS	P
Eosinophils	27	17	0.16
Immunoglobulin E	7	3	0.38
Interferon-gamma	7	17	0.16
GMCSF	10	17	0.23
TNF-alpha	13	27	0.11

*Results for all 30 subjects pooled. P values from Fisher's exact test. Results for each susceptibility group were also nonsignificant.

Nasal Lavage

Data was analyzed for nasal lavage sample measurements collected pre-exposure (B), 2 hr post-exposure (P2), and 22 hr post-exposure (P22) in FA and CAPs exposure conditions. Data capture was 75% for differential cell counts, and 95-99% for other variables. One analytical strategy applied to these data involved a model with susceptibility group, exposure atmosphere (CAPs vs. FA), and time (B vs. P2 vs. P22) as fixed factors, and subject as a random factor, including all

main effects and interactions of the fixed factors. If CAPs exposure affected subjects differently from FA, a significant atmosphere-by-time interaction should be found. If one susceptibility group was affected by CAPs more than another, a significant group-by-atmosphere-by-time interaction should be found. (However, power to detect these interactions was relatively low, given the relatively small number of subjects.) Where significant effects were found, follow-up analyses were performed including only the significant factors. Table 12 summarizes results from "conventional" models; results from "advanced" models were not substantially different.

Table 12. Significant (P < .05) Results from Mixed-Model Analyses of Nasal Lavage Assay Variables (Log-Transformed)

	Effect P-value				
Variable	Group	CAPs	Time	Interaction	Comment
Total cells			.004		increase from B to P2, partly reversed at P22
% Monocytes			.0003		decrease from B to P2, partly reversed at P22
% Lymphocytes					(nothing significant)
% PMNs			.0006		increase from B to P2, persists at P22
% Eosinophils			.03		increase from B to P2, partly reversed at P22
IgE					(nothing significant)
IgA				G*T .03	asthma(0) decreased, asthma(+) increased at P22, vs. earlier
IgM	.002				healthy > asthma(+) ~ asthma(0)
IgG4	.002			C*T .009	healthy > asthma(0) > asthma(+) down after FA, up after CAPS (see Figure 5)
IL4				G*T .009	asthma(0) up at P22, other groups down at P2 and P22, vs. B
IL5			.04		decrease from B to P2, persists at P22
IL8	.01				healthy > asthma(+) > asthma(0)
IFN gamma		.007			higher pre & post CAPs, vs. FA
TNF alpha	.006			G*C .004	asthma(+) lower, healthy higher with CAPS than FA, pre & post exposure

Variables that showed significant group main effects, i.e. overall differences between susceptibility groups, in nasal lavage fluid were IgM, IgG4, IL8, and TNF-alpha. By contrast, sputum variables that showed significant group differences were % monocytes, % PMNs, IgA, and IL4. Nasal lavage fluid total cell counts, % monocytes, % PMNs, and % eosinophils all showed significant main effects of time. Total cells, % PMNs, and % eosinophils increased from B to P2, while % monocytes declined, in both FA and CAPs exposures, possibly suggesting a nasal inflammatory response to the exposure protocol whether or not CAPs were present. These changes tended to persist at P22. Both IgA and IL4 in nasal lavage fluid showed significant group-by-time interactions, i.e. they changed differently with time in different susceptibility groups, unrelated to the presence of CAPs. The patterns of change for IgA and IL4 were different, however. Interferon-gamma and TNF-alpha showed significant main effects of atmosphere. These differences between CAPs and FA were present before as well as after

exposure, and so must be ascribed to chance preexisting differences rather than to actual effects of CAPs.

Only one nasal lavage variable, IgG4, showed a significant atmosphere-by-time interaction, i.e. a pattern of changes attributable to effects of CAPs. IgG4 tended to fall during and after FA exposure, but tended to rise (or fall less) during and after CAPs exposure. The pattern was reasonably consistent in all 3 susceptibility groups, even though their overall mean levels of IgG4 were different (highest in healthy, lowest in asthma-GSTM1-positive). These results are shown in Figure 12.

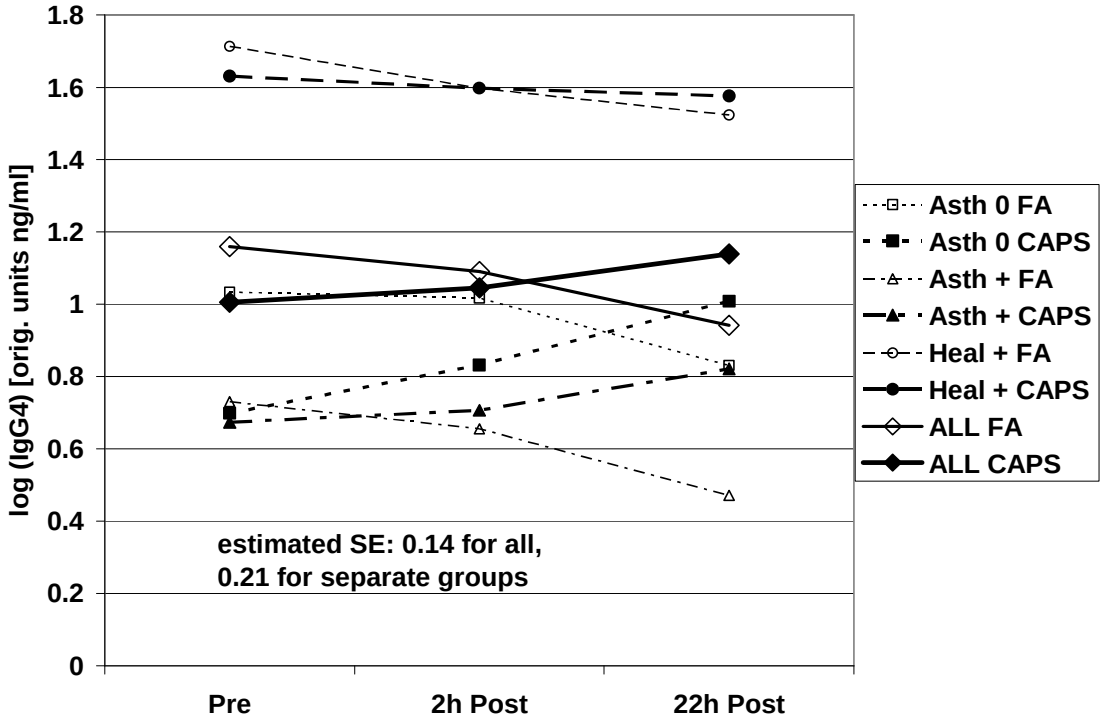

Figure 12. Estimated Mean Immunoglobulin G4 (Log-Transformed) in Nasal Lavage Fluid, as a Function of Time, by Group and Exposure Atmosphere

An alternative analytical strategy employed with nasal lavage data involved analyses of pre- to post-exposure changes in each variable, ignoring any pre-exposure differences. Results from an "advanced" model accounting for period, sequence, susceptibility group, and atmosphere effects corroborated the previous findings, showing IgG4 as the only variable that increased significantly after CAPs exposure, relative to FA.

Blood and Urine

Peripheral blood and urine samples were collected before exposure and the morning after exposure (times B, P22). Data capture was 92% for urine 8-isoprostane, 93% for serum IgG4, 95% for serum IgM, and 98-99% for other variables. Blood and urine data were first analyzed using a mixed model like that for nasal-lavage data, with 2 rather than 3 levels of the time factor. Except for vonWillebrand factor (vWF), original data distributions were markedly non-normal. Documentation of the nonnormal data distribution and log transformation results are included in

Appendix 2. Results from log-transformed data are given here (Table 13). Immunoglobulins E and G4 and C-reactive protein showed no significant variation by susceptibility group, atmosphere, or time. Serum IgA showed a significant time effect – a decrease (estimated mean 21%) over the approximately 24-hr interval between pre- and post-exposure measurements. Serum IgA also showed a significant main effect of exposure atmosphere (averaging higher in CAPs than in FA studies both before and after exposure) but no significant interaction, i.e. no change attributable to CAPs exposure. Serum IgG and IgM, plasma fibrinogen and factor VII, and urine 8-isoprostane all showed highly significant differences between susceptibility groups, but no significant variation by time or exposure atmosphere. GSTM1-null asthmatics were highest in IgG and fibrinogen; and lowest in factor VII, vWF, and 8-isoprostane. GSTM1-positive asthmatics were highest in vWF and lowest in IgG, IgM, and fibrinogen. Healthy subjects were highest in IgM, factor VII, and 8-isoprostane, and intermediate between the asthmatic groups for other variables.

Table 13. Significant (P < .05) Results from Mixed-Model Analyses of Blood and Urine Assay Variables

	Effect P-value				
Variable	Group	CAPs	Time	Interaction	Comment
Serum IgE (log)					(nothing significant)
Serum IgA (log)		.03	.006		decrease from B to P22, higher in CAPs than FA pre & post-exposure
Serum IgG (log)	<.0001				asthma(0) > healthy > asthma(+)
Serum IgM (log)	<.0001				healthy > asthma(0) > asthma(+)
Serum IgG4 (log)					(nothing significant)
Plasma CRP (log)					(nothing significant)
Plasma fibrinogen (log)	.0001				asthma(0) > healthy ~ asthma(+)
Plasma factor VII (log)	<.0001				healthy > asthma(+) ~ asthma(0)
Plasma vWF	.015				asthma(+) > healthy > asthma(0)
Urine 8-isoprostane	<.0001				healthy > asthma(+) > asthma(0)

Additional analyses were performed on the changes in blood and urine variables before to after exposure (differences P22-B). With untransformed data, distributions of these differences were still markedly nonnormal for some variables, so the following significant analytical results must be interpreted with caution: In a "conventional" model testing susceptibility group and exposure atmosphere effects, C-reactive protein showed a significant (P = 0.018) effect of CAPs, with nonsignificant group differences. The estimated mean changes were +7.5 ng/ml after FA and -1.8 ng/ml after CAPs – opposite to the expected direction. A Friedman rank-sum analysis – not assuming normal distribution – of C-reactive protein data also showed the CAPs effect as significant (P = 0.04) for all subjects pooled. (Similar Friedman analyses of other blood/urine data showed no significance.) An "advanced" model (testing effects of period as well as atmosphere) gave similar estimates to the “conventional” model, for the CAPs effect on C-reactive protein, and showed no significant difference by period. For IgG, the "conventional" model showed a significant (P = 0.025) interactive effect of susceptibility group and CAPs exposure: estimated mean changes in GSTM1-null asthmatics were +170 ng/ml after FA and -

3891 ng/ml after CAPs, while the other two groups showed small mean changes (-31 to -93 ng/ml) after both exposures. The "advanced" model for IgG showed no significant variation, although the negative change associated with CAPs approached significance (P = 0.07). The "advanced" model for IL8 showed a significant (P = 0.04) effect of period – the estimated mean change was more positive in second than in first exposure studies, regardless whether CAPs or FA was present. If data were log-transformed prior to taking the P22-B differences, CAPs-related effects on blood and urine measurements were all nonsignificant in the aforementioned analytical models. However, the effect of period (a more positive proportional change in second relative to first exposure studies) was significant not only for IL8, but also for IgE and 8-isoprostane. In summary, blood and urine changes from the initial collection just before exposure to the repeat collection on the morning following exposure showed equivocal evidence for CAPs effects, in the "wrong" direction according to the primary experimental hypothesis, as well as somewhat stronger evidence for a difference in response between the first and the second time in the exposure chamber.

Chemical Analyses of CAPS Samples

Data were obtained for elemental and organic carbon (EC, OC), various metallic and nonmetallic elements, and various polynuclear aromatic hydrocarbons (PAHs). PAH data were not analyzed statistically because most species were not detectable in most samples. For all 30 CAPs exposures, the mean and (standard deviation) of measured OC and EC concentrations were 32.4 (8.0) and 2.7 (1.9) $\mu g/m^3$ respectively. Neither OC nor EC concentration was significantly correlated with total mass concentration in CAPs exposures. In the 10 FA exposures in which particle samples were collected, the corresponding statistics were 21.1 (5.3) and 0.1 (0.2) $\mu g/m^3$ for OC and EC respectively. The differences between CAPs and FA exposures were significant, P < .01. These results are reasonably similar to previous findings in the same exposure chamber. The high OC levels in FA studies are believed to be generated by subjects. From a MIXED analysis intended to estimate the relative contributions of the subject and ambient air, by assuming that OC release differs between individuals but is consistent between exposures for a given individual, the mean concentration of ambient-air-derived OC in CAPs exposures was estimated as 12.7 $\mu g/m^3$.

Data from elemental analyses are difficult to interpret, due to several problems. These data were obtained by atomic absorption spectroscopy performed on acid extracts of the sample filters, in contrast to previous similar studies with elemental analysis by x-ray fluorescence. Concentrations are reported as ng/liter of extract, where the 50-ml extract represents 1.2 m^3 of air. The resulting calculated concentrations in the exposure atmosphere are a few ng/m^3 even for relatively abundant elements – far lower than expected. An exception is silicon, with readings orders of magnitude higher than any other element. After a query, the analytical laboratory staff rechecked the extraction procedure and concluded that the reaction vessels released substantial quantities of silicon into the extract. Thus, we judge that results for silicon are uninterpretable. We have analyzed other results in terms of the reported units – ng/l in extract – to estimate relative levels of different elements in CAPs and FA exposures. Table 14 presents summary statistics.

Table 14. Summary Statistics for Chemical Analyses of Particulate Samples from Exposure Studies (Blank Corrected)

Species	Units	CAPs		FA [Note A]	
		Mean	SD	Mean	SD
(total mass)	$\mu g/m^3$	186	43	34	17
EC	$\mu g/m^3$	2.7	1.9	0.1	0.2
OC	$\mu g/m^3$	32.4	8.0	21.1	5.3
Al	ng/l extract	30.1	16.7	13.5	5.7
K	ng/l extract	21.4	13.2	7.1	5.8
Ca	ng/l extract	34.7	20.2	18.8	5.1
Ti	ng/l extract	1.03	1.07	-0.23	0.47
V	ng/l extract	0.46	0.31	-0.06	0.07
Cr	ng/l extract	0.52	0.28	0.31	0.10
Fe	ng/l extract	36.3	19.1	7.2	3.7
Cu	ng/l extract	2.7	2.2	0.5	0.2
Zn	ng/l extract	8.0	3.5	3.0	0.7
Ba	ng/l extract	3.0	2.2	1.8	1.1
P	ng/l extract	3.6	7.0	1.7	6.0
S [Note B]	ng/l extract	158	90	9	35

A. 30 samples for total mass, 10 samples for individual species other than sulfur.
B. For sulfur, 19 CAPs samples and 7 FA samples were included in statistical analysis; other samples had unquantifiable concentrations due to high blank level.

Relationships of different elements in the CAPs samples were examined by calculating pairwise correlation coefficients, and by principal components analysis. Interpretability is limited by the small number of cases. A group of mostly crustal elements, including aluminum, potassium, calcium, titanium, chromium, iron, zinc, phosphorus, and barium, generally showed positive pairwise correlations and positive loadings on the first principal component, which accounted for 48% of overall variance. Copper and vanadium were generally uncorrelated with the aforementioned elements and with each other. Organic and elemental carbon correlated fairly strongly with each other, and to a lesser extent with most of the "crustal" elements. In general, concentrations of specific elements were not strongly correlated with total mass concentrations.

Exposure-response Analyses of Sputum Data

Table 15 shows correlation coefficients for pairs of exposure and response variables that showed a significant ($P<0.05$) relationship in at least one instance. (The Spearman rank correlation is used because of the nonnormal distributions of some variables.) Again, the small number of cases limits the ability to interpret either significant or nonsignificant results. The most suggestive results concern IgA and IgG4. They tended to increase with increasing mass concentration of CAPs (and also with chromium, the concentration of which was most positively correlated with mass concentration, among the elements measured).

Table 15. Pairwise Rank Correlations between Exposure and Sputum Response Variables

	Mass	Fe	Cu	Cr	Zr	Ba	% Lym	IL5	IgA
Fe	+.32								
Cu	+.24	+.47**							
Cr	+.64***	+.60**	+.31						
Zr	+.36	+.44*	+.15	+.35					
Ba	+.32	+.87***	+.39*	+.62***	+.54**				
% Lym	-.20	-.36	-.43*	-.28	-.11	-.32			
IL5	+.17	-.37*	-.02	-.12	-.30	-.40*	+.07		
IgA	+.41*	-.14	+.01	+.37*	-.13	-.14	+.07	+.19	
IgG4	+.43*	-.10	-.05	+.40*	-.06	+.02	+.04	+.02	+.68***

Notes: Variables that never showed significance ($P<0.05$) in an exposure-response relationship are not tabulated. Response variables and mass concentration were expressed as differences CAPs-FA; other exposure variables were expressed as concentrations in CAPs exposures. Additional significant correlations between response variables are described in text. *$P< 0.05$, **$P<0.01$, ***$P<0.001$

However, as Figure 13 shows, these modestly significant correlations depended on relatively few influential data points, while most subjects' differences between CAPs and FA were small or undetectable. Otherwise, IL5 was negatively correlated with two elements whose concentrations covaried strongly – iron and barium – and the percentage of lymphocytes was negatively correlated with copper. Although IgG and IgM showed no significant exposure-response relationships by this analysis, all the immunoglobulin differences (CAPs-FA) correlated positively with one another ($P<0.05$), thus demonstrating some degree of consistency in subjects' responses as determined by immunologic assays of induced sputum. Also, IL4 correlated positively with IL5, and IgA with IL8, when expressed as CAPs-FA differences.

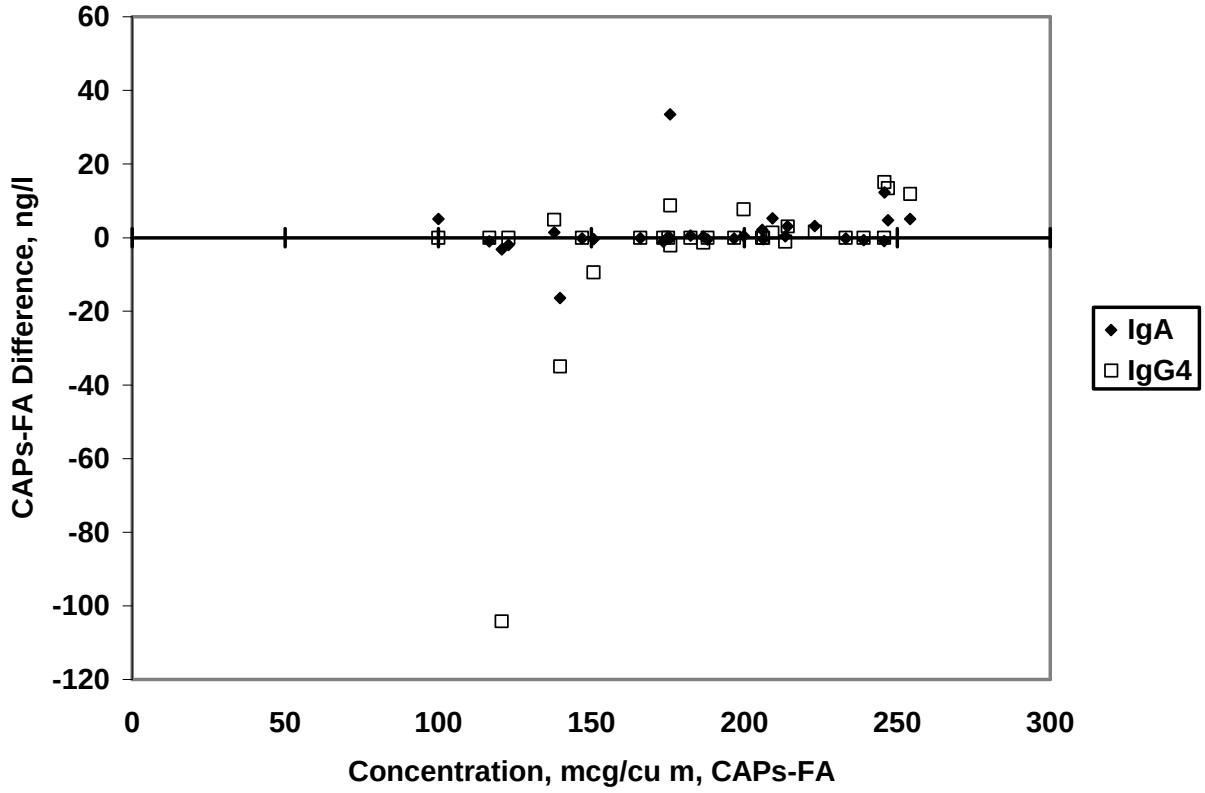

Figure 13. Difference in Post-Exposure Sputum Immunoglobulins, as a Function of Difference in Particle Mass Concentration, CAPs-FA

Cardiac Monitoring and Heart Rate Variability

The analytical strategy for the Holter electrocardiogram recordings was essentially the same as used for immunologic and physiologic data – mixed-model analysis with subject as a random factor (i.e., each subject as their own control) and with experimental conditions as fixed factors (i.e., comparing CAPs exposures against FA controls, ignoring variation in particle concentration and composition between individual exposure studies). Susceptibility group (healthy, asthmatic GSTM1-positive, or asthmatic GSTM1-null) was also included as a fixed factor in most models. When appropriate, data were subjected to log transformation before analysis. The data set supplied by US EPA included summary statistics for the entire 24-hr Holter ECG record, and for particular intervals (slices) including the 2-hr chamber exposure, a 4-hr interval post-exposure, a 16-hr interval post-exposure, an overnight interval (duration not specified), and 5-min intervals of enforced quiet rest preexposure (slice 'Pre') and during the follow-up exam the next morning, about 22 hr after the end of chamber exposure (slice 'P22'). Thus, for the 5-min slices a change from pre-exposure "baseline" to post-exposure could be estimated, and compared between CAPs and FA, by an atmosphere-by-time interaction term in the analytical model. For other intervals, no "baseline" was available so the comparison was only between CAPs and FA. In addition to conventional statistical models focusing on atmosphere or atmosphere-by-time effects, analyses were performed using "advanced" models that included tests for differences in response related

to period (first vs. second exposure study) and sequence (CAPs first or FA first). Period and sequence effects were generally nonsignificant, so conventional results are reported here.

Data capture was as low as 55% (for 5-min slices T-wave complexity) and as high as 77% (for 24-hr statistics), depending on the variable and time interval. Holter data were returned for only 23 of the 30 subjects, and certain intervals sometimes had unsatisfactory or missing data for some or all variables, e.g. because of a loose electrode, or because the subject came late to the follow-up examination and the recording ran out. The following variables were reported and have been analyzed for all intervals: mean R-R interval (RR), calculated heart rate (HR), standard deviation of normal beat-to-beat intervals (SDNN), and percentage of adjacent intervals different by >50 msec (pNN50). Many more variables were reported for the 5-min intervals. Results are reported here for the following variables, considered most important by the principal investigators: total power (TP); very low-, low-, and high-frequency power (VLF, LF, HF); normalized low- and high-frequency power (normLF, normHF); LF/HF ratio; median ST voltage from leads v2, v5, and II; various statistics concerning rate-corrected QT interval (QTcB); T amplitude; T complexity mean and standard deviation; TD/II norm variance; and TD/II variability index. In the majority of instances, these variables showed no significant ($P < 0.05$) differences between CAPs and FA atmospheres, between pre-exposure (Pre) and next-morning (P22) times of measurement, or between susceptibility groups (healthy, asthmatic GSTM1-positive, asthmatic GSTM1-null). Notable exceptions are described below.

A number of variables showed significant differences between Pre and P22 quiet-rest intervals (significant main effects of time), usually not significantly different between CAPs and FA. Table 16 summarizes these results. On mornings after exposure studies, heart rate was faster, heart rate variability was less by some measures, one measure of ST voltage was decreased, and some repolarization indices were altered, relative to the pre-exposure measurement about 24 hr earlier. Presumably this pattern of changes reflects an effect of the exposure-study experience per se, unrelated to the presence or absence of CAPs.

Table 16. Significant (P < 0.05) Main Effects of Time on Holter Variables (significant differences between pre and post-22-hr measurements regardless of atmosphere)

Variable	Estimated Mean (SE)		P Value
	Pre	P22	
Mean RR interval	880 (27)	833 (27)	0.009
Calculated HR	69.5 (2.1)	73.9 (2.1)	0.001
log (pNN50)	0.908 (0.163)	0.789 (0.162)	0.022
log (normalized high-frequency power)	1.665 (0.033)	1.584 (0.034)	0.048
Median ST voltage V5	41.8 (4.7)	35.6 (4.8)	0.005
Mean lead II QTcB*	400.0 (3.7)	405.1 (3.8)	0.018
T amplitude	245.5 (18.2)	219.1 (18.4)	0.005
log (SD QTcB)	0.910 (0.058)	1.065 (0.060)	0.019
log (TD/II norm variance)	-3.61 (0.10)	-3.18 (0.11)	0.003
TD/II variability index	-1.247 (0.101)	-0.888 (0.105)	0.001

*Mean lead II QTcB also showed significant differences between susceptibility groups (GSTM1+ asthmatics lowest) and between exposure days (lower both before and after CAPS). For other variables in table, only the time effect was significant. See Figure 14 concerning T-wave complexity.

In general, the 3 susceptibility groups were not significantly different. One exception concerned SDNN in 16-hr post-exposure intervals. Estimated geometric means were 102 msec for GSTM1 null asthmatics, 141 msec for GSTM1 positive asthmatics, and 95 msec for healthy subjects (P = 0.03 for group main effect). These differences were not significant for other time intervals. For the 2-hr intervals in the exposure chamber, pNN50 showed a marginally significant (P = 0.04) group difference; estimated geometric means were 4.0% for GSTM1 null, 2.8% for GSTM1 positive, and 0.8% for healthy subjects.

There was a near-significant (P = 0.06) increase in mean RR interval during chamber exposures to CAPs relative to FA (estimated means 633 and 614 msec respectively), and a corresponding near-significant (P = 0.08) decrease in calculated HR (estimated means 95 during CAPs, 98 during FA exposures). Interpretation of these differences is difficult in that they reflect 4 successive cycles of exercise and rest, and slight differences in exercise intensity between exposures cannot be ruled out. Nevertheless, HR was a slightly slower overall during CAPs exposures. Stronger statistical evidence for an effect of CAPs exposure came from T-wave complexity data for 5-min slices. Figure 14 shows these results. The raw data consist of a mean and a standard deviation of T-wave complexity for each individual at each time. Because distributions were skewed, both the mean and the SD values were log-transformed for analysis. Results are graphed in the original units, i.e. as geometric means. In light of the relationship between the 2 variables, the estimates of T-complexity SD are plotted as "error bars" on the bar graphs of T-complexity mean, although they do not represent confidence limits in the usual sense. Both mean and SD increased noticeably at P22 after FA exposure, but much less so after CAPs exposure. For T-complexity mean, P (time) = 0.024 and P (atmosphere-by-time) = 0.07; for T-complexity SD, P (time) = 0.006 and P (atmosphere-by-time) = 0.019.

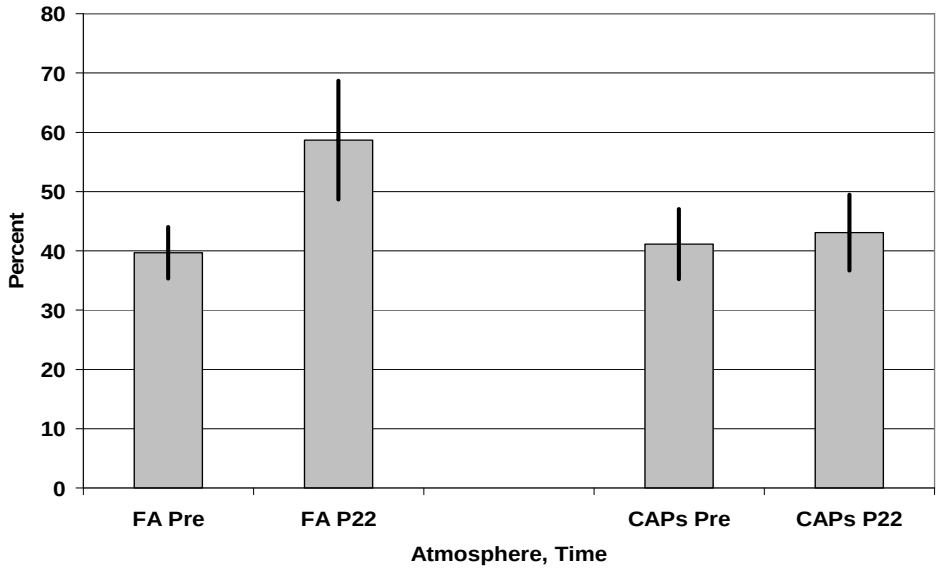

Figure 14. T-wave Complexity (results from mixed-model analyses of log-transformed data). Large gray bars: Estimated geometric means of individual mean T-wave complexity values, by atmosphere and time. Small black bars: Estimated geometric means of individual standard deviations of T-wave complexity. See text for explanation.

DISCUSSION

Particulate air pollution exposure has clearly been linked with adverse health outcomes. This association has been demonstrated by large epidemiologic studies and supported by some albeit fewer prospective controlled exposure studies with diesel exhaust particles and CAP. The primary objectives of this prospective human exposure study was to test the hypothesis that subjects with specific "susceptibility" factors for the pro-inflammatory effects of particulate air pollution would demonstrate heightened respiratory and/or systemic inflammatory responses to concentrated air particle (CAPS) exposure. The susceptibility factors chosen for study were pre-existing asthma and GSTM-1 null polymorphism. Based on these factors, three specific populations were enrolled to undergo a randomized single-blinded cross-over exposure protocol with CAPS and a control exposure of filtered air (FA). The three populations of interest for comparison were "high-risk" (asthma and GSTM1null), "moderate-risk" (asthma and GSTM1present), and "low-risk" (healthy and GSTM1positive). A number of physiologic and biologic endpoints were measured for comparison to investigate the effects of CAPS exposure compared to FA and determine differential effects, if any, between these preselected groups of human subject.

The preponderance of data from the study, including a wide array of physiologic and immunologic endpoints, fails to demonstrate clear robust differences between the exposure responses of high vs. moderate vs. low-risk subjects. In addition, with the exception of an increase in FeNO for all groups, CAPS exposure as performed in this study does not appear to produce a robust inflammatory respiratory or systemic response in human subjects, regardless of the presence or absence of the aforementioned risk factors. A number of potentially interesting observations emerge from the statistical analysis, however the lack of concurrent supporting inflammatory biomarkers in specific populations or exposure conditions casts doubt about the reliability of such findings. Essentially, our study did not find that subjects with asthma and/or GSTM1 null genotype were more susceptible to the inflammatory effects of CAPS exposure. Certainly, this conclusion is subject to a number of study limitations which will be addressed below.

Significant differences between susceptibility groups were seen in baseline FeNO for asthmatic vs. healthy subjects and a trend for decreased FEV1/FVC is asthmatics vs. healthy subjects. These findings are as expected in the preselected groups given the increased pulmonary inflammation and obstruction associated with asthma. However, the fact that the FEV1/FVC ratio was not statistically different between asthmatic and healthy subjects speaks to the mild nature of the asthma population enrolled in the study.

While some physiologic endpoints showed changes with the exposure procedures, few differences were seen in responses to exposure conditions when comparing FA vs. CAPS, or when comparing responses between susceptibility groups. Systolic blood pressure (SBP) showed a significant overall drop with little difference between CAPS and FA. This SBP change may be attributable to the effect of exercise. While SBP generally increases during exercise in healthy subjects, post-exposure BP readings were obtained during the post-exertional period when decreases in SBP are well-documented in healthy human subjects.[95] Group differences were non-significant in most analytical models, though GSTM1null subjects showed a

significantly smaller loss in SBP compared to GSTM1-positive subjects in one model. Previous studies by Gong et al. have demonstrated decreased SBP in asthmatics and increases in healthy subjects with CAPS exposure, though GSTM1 status was not known in the reported study population.[35] Subject-reported symptoms increased significantly during and after exposures for GSTM1null asthmatics compared to GSTM1present subjects. However, these results did not show significant differences for FA vs. CAPS exposures so it is difficult to attribute this apparent symptomatic worsening to biologic CAPS effects. One could hypothesize an inflammatory or nonspecific irritant effect of the general exposure procedure itself that may preferentially affect GSTM1null subjects, but in the absence of corroborating objective biomarker data, it is difficult to interpret the significance this subjective endpoint. Additionally, the observed reduction in symptom score associated with NO2 exposure concentration, while statistically significant, is unlikely to represent a causative association. Chamber studies of NO2 effects at concentrations higher than observed here show little effect on symptoms.[80] A favorable effect of NO2 or any unmeasured pollutant that covaried with NO2 on symptoms is intuitively unlikely.

Increases in exhaled nitric oxide (FeNO) were the only statistically significant biologic change attributable to CAPS exposure compared to FA in our study. This effect did not vary significantly according to asthma or GSTM1 status, though the mean CAPS-FA difference was larger in asthmatic compared to healthy subjects. Though a number of observational studies have documented an association of particle exposure with increased FeNO in both healthy and asthmatic populations[96-98] the present finding of increased FeNO in both asthmatic and healthy subjects after fine CAPS exposures has not been previously described. Previous studies of similar subjects exposed to fine CAPS[36,99] did not include FeNO measurements, so no directly comparable earlier data exist. Indirectly comparable data are available from studies of coarse CAPS,[35] ultrafine CAPS,[100] and diesel exhaust (report in preparation). None of these showed consistent increases in FeNO after exposure. In previous studies, healthy subjects tended to increase FeNO after ultrafine CAPS exposures, like the present group; but asthmatics tended to decrease, unlike the present group. Thus, this finding may represent confirmation of FeNO as a useful marker of CAPS-induced pulmonary inflammation. It is, however, notable that few other inflammatory changes were observed or attributable to CAPS exposure. This may cast doubt on the reliability of the FeNO finding or alternatively indicate that FeNO is a more sensitive marker of respiratory inflammation than other biomarkers in this setting. Should the latter be demonstrated in future studies, FeNO may represent a useful diagnostic tool to screen for the inflammatory effects of fine particulate exposure both in healthy and asthmatic individuals.

Some sputum parameters appeared to be affected by susceptibility group, however were not significantly affected by exposure conditions (CAPS vs. FA). GSTM1-positive asthmatics had significantly higher levels of sputum PMN counts and lower sputum monocyte counts compared to other groups. The presence of increased sputum neutrophils is found in a subset of asthmatics, though sputum eosinophilia is a more common finding. The explanation for this finding in GSTM1-positive vs. GSTM1-null asthmatics is not clear. By comparison, a previous exposure study of healthy human subjects reported a mild increase in bronchoalveolar lavage neutrophils with CAPS exposure relative to FA.[101]

Sputum IgA was also higher in GSTM1-positive asthmatics in the study, regardless of exposure condition. Increased specific allergen and antigen IgA levels have been reported previously for atopic asthmatics compared to healthy controls.[102] However, no clear explanation exists for the

association of elevated sputum IgA with GSTM-present genotype. Interestingly, additional analyses demonstrated significant group differences for sputum IgA when CAPS-FA was considered. Healthy subjects showed a significant increase while GSTM1-positive asthmatics showed little change and GSTM-null asthmatics showed a mild decrease with CAPS exposure. This overall group variation with CAPS exposure approached statistical significance ($p=0.06$). With the recently recognized anti-inflammatory role of mucosal and systemic IgA,[103,104] it is interesting to consider possible links between IgA and the anti-oxidant role of GSTM1 that would potentially explain this association. Such theories would be speculative and require considerable additional study to investigate the association and/or mechanism.

Additionally, sputum IL-4 showed effects of susceptibility group and exposure condition though it must be recognized that some subjects (3 healthy, 2 GSTM-null asthma, 1 GSTM-present asthma) had no detectable IL-4 in sputum at any time point. Asthmatic groups had higher mean sputum IL-4 levels compared to the healthy controls. This is not an unexpected finding since IL-4, as a predominant Th2 cytokine, is known to be increased in the airway of many patients with asthma, particularly asthmatics with atopic and/or eosinophilic asthma. Surprisingly, however, all groups showed decreases in mean IL-4 levels after either exposure (CAPS or FA) relative to baseline levels. This outcome contrasts with previous data from DEP studies suggesting that particulate air pollutants induce increased IL-4 production from T-cells. The observed decrease in sputum IL-4 was seen with both CAPS and FA exposure implying this is not a particulate effect but some other procedural factor, though to date this observation is unexplained.

Collection and analysis of nasal lavage samples in this population was an additional step that has not commonly been included in exposure chamber studies. Previous human studies at our institution have demonstrated a number of the inflammatory and proallergic effects of DEP in the upper airway as well as the importance of GSTM1 in modifying this inflammatory response to particulate pollution. Thus, we felt this was an important component in investigating the responses to CAPS in these susceptible populations. Group differences were demonstrated in a number of biomarkers including IgM, IgG4, IL-8, and TNF-alpha as illustrated in Table 12. These do not show a clear pattern with regard to group comparisons, i.e. no group showed consistent elevations across a number of antibody or cytokine endpoints. Thus, it is difficult to arrive at any conclusions regarding the significance of these differences. Additionally, the NL differences between groups do not appear to correlate with group differences in sputum variables. This may be due to variation in upper and lower airway inflammation within subjects or non-correlation for other technical reasons such as collection technique. NL cell counts, %PMNs, and %eosinophils all increased from baseline to P2 and tended to persist at P22, suggesting a nasal inflammatory effect from the exposure protocol, however this occurred whether CAPS was present or not. Only nasal IgG4 appeared to be significantly impacted by CAPS exposure. Changes in NL IgG4 levels with CAPS exposure were significantly different compared to FA exposure, generally with increases post-CAPS and decreases post-FA. Levels were highest in the healthy group, but the changes with FA vs. CAPS were generally consistent among the 3 susceptibility groups. IgG4 has previously been reported to be high in allergic subjects[105] and there is considerable evidence that IgE and IgG4 share common molecular regulatory mechanisms.[106,107] IgG4 is also believed to play a role in down-regulation of specific IgE responses by acting as a “blocking antibody”. Previous work with DEP, environmental tobacco smoke, and other particulates have demonstrated increases in mucosal IgE production,

however we did not see increases in mucosal IgE with CAPS exposures. Thus, the presence of higher mucosal IgG4 levels in nonatopic, nonasthmatic subjects and the induction of IgG4 in the upper airway by CAPS is somewhat puzzling, but could represent an immunoregulatory and anti-inflammatory finding. While sputum IgG4 levels were considerably higher in the non-asthmatic group, the CAPS-exposure upregulation of IgG4 was not replicated in the sputum data.

Results from biomarkers in blood and urine samples were disappointing in that they did not show any exposure differences attributable to CAPS. Previous human studies of CAPS exposure effects on various serologic biomarkers have yielded variable results from no significant change[108] to mild increases in fibrinogen[101], D-dimer and IL-8[109], though the latter two effects were observed with concentrated ultrafine particles specifically. In the current study, serum IgA levels were higher both pre- and post- CAPS exposure compared to FA, so the difference was not attributable to CAPS exposure. Group differences were seen between serum IgG and IgM, plasma fibrinogen and factor VII, and urine 8-isoprostane but the biologic significance of these differences is unclear. For example, urine 8-isoprostane levels were highest in the healthy subjects which is somewhat unexpected, though urinary 8-isoprostane levels are known to be highly variable is asthmatics. No significant variation of the biomarkers was induced by exposure atmosphere. Analysis of normalized data failed to demonstrate any inflammatory changes in blood or urine samples.

HRV is increasingly recognized as an important cardiovascular outcome that may indicate adverse health effects of air pollutant exposure. Reduced HRV is considered a prognostic marker for the development of cardiac arrhythmia. Epidemiological studies have demonstrated associations of air pollution exposure with decreased HRV,[110-112] while controlled particle exposure study results have showed variable cardiovascular effects though generally suggesting decreases in at least some HRV measures.[35-36,81,94,100,113-115] Careful analysis of the Holter electrocardiographic data in the present study demonstrated a number of cardiovascular variables with statistically significant changes from pre- to post-exposure measurements. These included increased HR and indicators of decreased HRV post-exposure as has been reported in other particle exposure studies referenced above. However, in the current protocol, these changes were observed across all groups regardless of exposure conditions (CAPS or FA), supporting an effect of the general exposure procedures rather than a specific particle effect. We observed significant differences in SDNN at 16-hr post-exposure intervals between susceptibility groups ($P = 0.03$ for group effect), with the highest observed values for GSTM1 positive asthmatics and the lowest values for healthy controls. However, this difference was independent of exposure atmosphere and was not significant for other time intervals. Likewise, pNN50 for the 2-hour intervals in the exposure chamber showed a significant difference ($P = 0.04$) between groups, but is not attributable to CAPS exposure. Previous exposure studies have demonstrated similar decreased HRV endpoints (i.e. SDNN, pNN50) associated with particle exposure,[35,117] particularly in healthy subjects. Our study data shows decreased HR variability measures in the healthy subjects regardless of the exposure conditions.

HRV changes that appeared attributable to CAPS exposure across all groups included a mild decrease in HR and decreased T-wave complexity and variability. This result is inconsistent with previous reports of increases in both HR and T-wave complexity/variability after particle exposure,[35, 118-119] though some previous studies have differed from our protocol with regard to

particle size (ultrafine) and study population (ischemic heart disease). Thus, it is difficult to directly compare study results. However, our CAPS exposure data appears to be opposite the predicted effect on HRV, given an expected stress response to CAPS. It is possible that CAPs exposure tended to inhibit subjects from exercising as vigorously compared to FA, leading to the slightly decreased HR during exposures. The T-wave complexity changes are more difficult to interpret. While exercise is expected in increase T-wave complexity acutely,[120] the effect of exercise on T-wave complexity and variability at 24-post is largely unknown. Our study data suggests CAPS exposure blunted a post-exposure increase in T-wave complexity/variability seen with FA. As increased T-wave complexity/variability indicates disturbance of normal electrical repolarization, prevention of the increase might seem to be a favorable effect of CAPS exposure. This is unlikely in principle, given the known adverse health effects of CAPS, so the most plausible explanation is that this result represents a "chance" finding of statistical significance among many tests. These CAPS-attributable HRV findings were not different between susceptibility groups.

A major consideration in air pollutant exposure studies is characterization of the exposure conditions in order to understand, as is technically feasible, the quality and quantity (or dose) of components in the respiratory exposure. Our study employed well-established protocols and equipment which have been used successfully for a number of previous exposure studies. Air monitoring results showed that the experimental exposures to concentrated fine ambient particles were reasonably close to the target concentration of 200 $\mu g/m^3$. The new type of continuous-monitoring nephelometer used in this project provided only limited improvement in accuracy (as judged by gravimetric measurements). This monitoring uncertainty, along with unpredictability of atmospheric conditions and limitations of particle concentrator performance, resulted in appreciable variability of exposure levels between subjects. The same was true of previous CAPS exposure studies here and elsewhere. One potential issue with regard to the exposures is the characteristics of the CAPS. Though EC/OC results appear relatively consistent with previous CAPS exposures at our site, the PAH levels detected in the particles appear relatively low, given many filter samples that were below the limit of detection for a number PAHs. Thus, if particle redox activity is strongly correlated with PAH content, it is possible this apparent reduced PAH content may be a contributing factor to our findings. Particle analysis demonstrated detectable levels of a number of transition metals and elements believed to be important in the generation of ROS and inflammation, though again, these levels were considerably lower than expected. Though we have no reason to believe the collected air particles in our region have changed substantially compared to previous studies at our site, qualitatively, the chemical composition of CAPS used in our study may differ from those used in other CAPS exposure studies.

With regard to specific exposure factor correlation with biologic endpoints, there was little data to support specific factors leading to an inflammatory response. Sputum IgA and IgG4 were most closely correlated with mass concentration of CAPs and chromium (which positively correlated with mass concentration). These sputum antibodies tended to increase with increasing mass concentration; however this trend was strongly influenced by a few data points. Sputum IL-5 was negatively correlated with iron and barium exposure and percentage of sputum lymphocytes was negatively correlated with copper.

From the current study, there is little data to support the hypothesis that "high-risk" subgroups, as defined by the presence of asthma and GSTM1-null genotype, are more susceptible to the inflammatory effects of CAPS exposure. We saw no differential respiratory or systemic inflammatory changes between groups attributable to CAPS exposure after measurement of spirometry, FeNO, sputum/nasal lavage inflammatory markers, and HRV.

Conclusions from the current study are tempered by several limitations. First, the individuals enrolled in the asthma groups were mild-moderate with regard to their clinical status. Subjects were not taking inhaled or systemic corticosteroids and were required to have baseline FEV1 >70% to enroll in the exposure protocol. For safety and ethical reasons, we were unable to study a more severe asthma population. The inflammatory and symptomatic differences as well as susceptibility to pro-inflammatory effects between the asthma and healthy groups may have demonstrated more significant differences with the enrollment of more clinically severe asthma subjects.

Second, the strength of the conclusion is limited by study power. The current study was designed to detect a 3% exposure-related reduction in FEV1 (considered the smallest clinically meaningful FEV1 change) with power of 0.8 using a one-tail test with alpha = 0.05 and N = 10. (No directly relevant information was available to calculate power to detect the immunologic effects of most interest; but detectable FEV1 changes would imply prior or concurrent immunologic changes large enough to detect.) Thus, while we can be reasonably confident that this degree of FEV1 change has not occurred in the groups investigated, a number of more subtle changes in biomarkers could have potentially escaped detection. To address that issue, post-hoc power calculations were performed for selected sputum immunologic variables, based on standard deviations of individual CAPs-FA differences in log-transformed data. Results indicated that, for N = 10, one-tail alpha = 0.05, and power = 0.8, a 1.7-fold increase in sputum IgA or IgM, or a 3.5-fold increase in IgG or IL-8, could have been detected. Larger changes – sometimes 10-fold or more – are observable in some immunologic studies, e.g. nasal challenges with allergen and diesel particles. Thus, our investigation's power to detect immunologic responses was reasonably good, but perhaps not optimal for detection of subtle effects.

A third potential limitation is that variability in exposure conditions as discussed in the previous section may contribute to some inconsistencies or variability in subject responses. Individual response to PM exposure may vary by season or other time dependent factors (e.g. day of week) because of variations in biology, emissions, or atmospheric chemistry. While such effects have been examined in very large observational studies, the influence of such factors was outside the scope of the current study design. This study is unable to address seasonal or day-of-week effects, since by nature it involves a small population studied at irregular intervals over multiple seasons, on weekdays selected arbitrarily for volunteer availability and suitable atmospheric conditions. As mentioned previously, selection of suitable atmospheric conditions for CAPs exposures potentially introduces confounding by other factors that vary with ambient PM. One such factor that could be measured, NO_2, showed a significant dose-response relationship with symptoms, but not in a direction suggesting unfavorable response to either NO_2 or PM. Another aspect of exposure variability is the sometimes appreciable concentration of particles in filtered air. Given that these particles are mostly too large to be inhaled into the lower respiratory tract, and are predominantly generated from the subject's body surface and clothing, they should not

represent an important interference. Nevertheless, they illustrate an inherent limitation of this and most other controlled exposure studies: the air exchange rate in the subject's personal environment is lower in the exposure chamber than it would be outdoors, even at times of low wind speed. Thus, the subject's own emissions add to the inhalation exposure to a greater extent than in the "real world", and at least in principle, they might either exacerbate or mask the exposure effects being studied. In CAPs studies, because of the technical difficulty of large-scale particle concentration, both the limitation of air exchange rate and the degree of confinement in the exposure chamber (with possible attendant psychological stress influencing responses) are more severe than in typical exposure studies with gas-phase or artificially-generated particulate pollutants.

A fourth limitation concerns the many response variables being measured in relatively few subjects, whose personal environmental stresses outside the confines of the experiment may affect their responses. This design makes it likely that spurious statistically significant differences will be found, either because of uncontrolled and unmeasured intercurrent interferences, or because a few "significant" differences must be found by chance in any large collection of statistical test results. In ideal circumstances with truly zero effect of the experimental factor upon any of a large number of mutually independent response variables, with alpha = 0.05 about 5% of the statistical results should appear significant, with about half of those in the "right" direction and half in the "wrong" direction according to the experimental hypothesis. In this study, most response variables are undoubtedly related with others, but the strength of these relationships is uncertain, so that the expected incidence of spurious significant results differs from the ideal to an uncertain degree. Given that CAPs-FA differences with $P < 0.05$ were few and scattered, we cannot distinguish between the alternative possibilities that all those results are spurious, or that at least some of them are causal and reflect health risks from ambient PM. The same might be said for previous CAPs studies in this laboratory, and possibly others as well.

Fifth, biologic heterogeneity between human individuals undoubtedly affects results particularly with immunological assays where both inter- and intra-subject variability over time may be considerable. Large biologic variations due to age, diet, genetic background, activity level, ambient exposures, and disease history are difficult to control for and can influence measured outcomes. Our study was not designed to examine the impact of these various factors on response to CAPS exposure, nor were we able to collect data in many of these domains. Therefore any analyses examining these factors would be post-hoc in nature and limited by data collection and sample size. Obesity is an emerging factor with potential impact on individual response to particulate matter[121, 122], but was not included in the original hypotheses for the project nor were subjects selected based on such criteria. However, susceptibility groups had similar numbers of overweight or obese subjects (7 asthmatic GSTM1 null, 7 asthmatic GSTM1 present, 8 healthy GSTM1 present), so this factor is unlikely to influence the reported between-group comparisons.

Finally, while significant evidence supports GSTM1 as a central antioxidant enzyme in protecting airway epithelium and other cells, a number of other Phase II antioxidant enzymes and other cytoprotective mechanisms play a role in reducing cellular oxidative stress.[123] It was our hypothesis that this genetic polymorphism would predict susceptibility and increased

inflammatory responses to CAPS exposure. However, it would seem, based on our study results, that important co-factors, genetic or otherwise, may modulate the response to particle exposure or oxidative stress in the absence of GSTM1. Thus, for future studies, it may be important to consider additional defined genetic polymorphisms in subject selection so as to determine the influence of an "antioxidant network" that influences the host response to pro-inflammatory particulates.

SUMMARY AND CONCLUSIONS

This prospective human exposure study was conducted to test the hypothesis that individuals with certain susceptibility factors have heightened inflammatory and airway responses to PM exposure. Susceptibility factors of interest were subjects with asthma and subjects with GSTM1-null genotype. We performed a single-blind randomized dose crossover study with volunteer human subjects undergoing controlled exposures to filtered air (FA), and concentrated ambient particles (CAPS). Ten mild- moderate asthmatic GSTM1 null subjects, 10 mild- moderate asthmatic GSTM1 present subjects, and 10 healthy GSTM1 present subjects were enrolled to determine the short-term effects of CAPS exposure in individuals likely to be at risk for adverse effects. Outcome measures included symptom scores, physiologic measures (vital signs, spirometry, exhaled nitric oxide, heart rate variability) as well as serum, sputum, and nasal lavage samples for inflammatory biomarkers. The target particle mass concentration for CAPS exposures was 200$\mu g/m^3$ for 2 hours with subjects performing submaximal exercise for 15 minutes of every half-hour. The major findings of the study included:

- Particle mass concentrations averaged 187$\mu g/m^3$ for CAPS and 35 $\mu g/m^3$for FA during the 2-hour exposures.
- Few significant CAPS-attributable changes were observed for physiologic and symptom endpoints, consistent with findings in previous studies using similar exposures.
- An unequivocally significant relative increase in FeNO was associated with CAPS exposure for all groups, without significant changes in most other concurrent respiratory or systemic inflammatory markers.
- Sputum total cell counts trended higher after CAPS than after FA exposures and nasal lavage IgG4 was increased after CAPS and decreased after FA exposure for the entire population.
- GSTM1-null asthmatics reported increased symptom scores during both CAPS and FA exposures.
- Post–exposure systolic blood pressure decreases were observed in all groups for both FA and CAPS exposures.
- Increased heart rate and decreased HRV post-exposure was observed across all susceptibility groups regardless of exposure conditions (CAPS or FA).
- Minimal HRV changes were attributable to CAPS exposure or susceptibility group.
- Overall, some evidence to support the hypothesis of airway inflammatory responses to CAPS exposure, but these responses were not significantly different between subject groups.

In conclusion, the current data from this study does not support the hypothesis that human subjects with mild-moderate asthma or GSTM1-null genotype have greater inflammatory responses to short-term CAPS exposure at levels approximating 200$\mu g/m^3$ for 2 hours. If such responses are influenced by asthma status and GSTM1 genotype, the influences appear to be subtle and were not detected by the instituted study design.

RECOMMENDATIONS

Future studies in this important area of individual host susceptibility to the pro-inflammatory effects of CAPS should consider the following in order to maximize detection of important differences in response: 1) larger-scale experiments with increased power 2) alternatives to spirometric changes as primary endpoints 3) increased CAPS exposure (higher concentration and/or greater duration), 4) ethical inclusion of more clinically severe asthmatics, 5) consideration of additional genetic and host co-factors (i.e. dietary) that may modulate inflammatory response to oxidative stress. Additionally, the study demonstrates a relative increase in FeNO with CAPS exposure suggesting a potential role for this measurement as an early sensitive marker of airway inflammatory responses to fine particles in both healthy and asthmatic individuals. Inclusion of FeNO measurement in future fine CAPS exposures will be useful in determining the significance of this finding.

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GLOSSARY OF TERMS, ABBREVIATIONS, AND SYMBOLS

AP-1: activator protein-1
ARB: Air Resource Board
ATS: American Thoracic Society
B: baseline
BP: blood pressure
BSA: bovine serum albumin
CAPS: concentrated ambient particles (here, fine particles <2.5 µm mass median aerodynamic diameter)
CE-CERT: Center for Environmental Research & Technology
CO: carbon monoxide
DE: diesel exhaust
DEP: diesel exhaust particles
DNA: Deoxyribonucleic acid
EC: elemental carbon
ECG: electrocardiogram
EIA: enzyme immunoassay
ELISA: enzyme-linked immunosorbant assay
EPA: Environmental Protection Agency
ETS: environmental tobacco smoke
FA: filtered air
FeCO: fractional concentration of exhaled CO
FeNO: fractional concentration of exhaled NO
FEV_1: forced expired volume in one second
FVC: forced vital capacity
GM-CSF: granulocyte macrophage colony-stimulating factor
GSTM1: glutathione-S-transferase mu 1 gene
GSTP1: glutathione-S-transferase pi 1 gene
HEPA: high efficiency particulate air
HF: high frequency
HR: heart rate
HRV: heart rate variability
IFN-γ: interferon-gamma
Ig: immunoglobulin
IL: interleukin
LAREI: Los Amigos Research and Education Institute, based at RLANRC
LF: low frequency
mL: milliliter
MOUDI: micro-orifice uniform-deposit impactor
NAEPP: National Asthma Education and Prevention Program
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NIST: National Institute of Standards and Technology
NL: nasal lavage
normLF: normalized low-frequency
normHF: normalized high-frequency

NSBH: non-specific bronchial hyperreactivity
NO: nitric oxide
NO_2: nitrogen dioxide
OC: organic carbon
P2: 2 hours post-exposure
P22: 22 hours post-exposure
PAH: polycyclic aromatic hydrocarbon
PBS: phosphate buffered saline
PEF: peak expiratory flow
PM: particulate matter
PMNs: Polymorphonuclear leukocytes
pNN50: percentage of adjacent intervals different by >50 msec
PPB: parts per billion
RFLP: restriction fragment length polymorphism
RLANRC: Rancho Los Amigos National Rehabilitation Center
ROS: reactive oxygen species
RR: R to R interval
SaO_2: arterial oxygen saturation (% oxyhemoglobin)
SBP: systolic blood pressure
SDNN: standard deviation of normal beat-to-beat intervals
SO_2: sulfur dioxide
Th2: T-helper Type 2 cells
TNF-α: tumor necrosis factor-alpha
TP: total power
UCLA: University of California, Los Angeles
VLF: very-low frequency
vWF: von Willebrand factor
WT: wild-type

Appendix 1

[**Gong et al. Concentrated Particle Exposures: Additional Methodological Details and Results** from **Health Effects Institute Report 2003: Controlled Exposures of Health and Asthmatic Volunteers to Concentrated Ambient Particles in Metropolitan Los Angeles]**

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APPENDIX AVAILABLE ON REQUEST

Research Report 118

Controlled Exposures of Healthy and Asthmatic Volunteers to Concentrated Ambient Particles in Metropolitan Los Angeles

Appendix D. Concentrated Particle Exposures: Additional Details of Methodology and Results

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APPENDIX D. CONCENTRATED PARTICLE EXPOSURES: ADDITIONAL DETAILS OF METHODOLOGY AND RESULTS

CONCENTRATOR AND EXPOSURE CHAMBER

The concentrator was manufactured and installed under the direction of Harvard School of Public Health staff, with components like those employed at Harvard. Figure D-1 presents a schematic illustration of the concentrator, exposure chamber, and monitoring instruments. The first element of the concentrator system is a model TE-6001 size-selective inlet (Tisch Environmental, Inc., Cleveland, OH), mounted on the outside of the laboratory structure just above roof level. This unit, similar to those used on high-volume air samplers, is designed to exclude particles >2.5 µm in aerodynamic diameter. Ambient air is drawn in at 5000 L/min by a single high-capacity main pump. The air then passes through a stainless-steel duct to the laboratory interior, and through a tapered stainless-steel transition piece into concentrator stage 1, consisting of 5 virtual impactors (slits) in parallel (see Figure D-2). The major flow (800 L/min from each impactor), depleted of particles, is drawn off laterally by the main pump. The minor flow (200 L/min each slit, 1000 L/min total), enriched in particles, proceeds longitudinally to concentrator stage 2, which consists of one slit like those of stage 1. Here again, the major flow of approximately 800 L/min is drawn off laterally by the main pump, and the particle-enriched minor flow (170-200 L/min, typically 180 L/min) is drawn through a straight 3-inch (7.6-mm) diameter pipe (chamber inlet pipe), into the front and out the rear of the exposure chamber. An auxiliary pump downstream of the exposure chamber, along with 4 small pumps to supply air sampling instruments, provides this minor flow. The chamber inlet pipe is interrupted by a gate valve that can divert any proportion of the flow through a parallel path incorporating a high-efficiency particulate (HEPA) filter. For filtered-air (FA) exposure studies, the gate valve is fully closed and the entire air flow to the chamber passes through the HEPA filter. For concentrated ambient particle (CAP) exposure studies at times of relatively low ambient pollution, the gate valve is fully open and the entire output of the concentrator flows directly through the chamber. At times of moderate to high ambient pollution, the gate valve is partially closed so that part of the air flow is filtered, sufficient to keep the particle concentration delivered to the chamber near the target of 200 $\mu g/m^3$, 2-hr time-weighted average (TWA). Valve adjustment is guided by real-time concentration readings from the Data RAM nephelometer sampling chamber air, taking account of artifacts related to relative humidity. (Both ambient and in-chamber temperature and relative humidity are monitored during exposure periods with portable electronic instruments.) During typical morning exposures, the in-chamber concentration profile is allowed to follow (roughly speaking) that in ambient air. Accordingly, the chamber concentration may be well above 200 $\mu g/m^3$ early in the exposure (400 $\mu g/m^3$ is considered the short-term exposure limit) and well below 200 $\mu g/m^3$ later. This approach is necessary to approximate the correct TWA consistently, given that ambient particle concentrations may decline appreciably during midmorning.

The only major problem encountered with concentrator operation has been fouling of impactor slits with particles, resulting in increased pressure drop across the slits and decreased concentration efficiency. As was the case in the Harvard dog studies (Godleski et al 2000), this tended to occur during periods of high particulate pollution combined with high humidity. Although the fouling observed in the Harvard environment was a more gradual process, in our circumstances it sometimes became noticeable within one hour of starting the concentrator, even though slits were cleaned prior to every study. To deal with the problem, whenever pressure drop in the chamber/downstream of stage 2 reached 18 in H_2O (46 cm H_2O) - twice its optimum value - major flow was decreased as necessary, up to 25%, to decrease the pressure drop. The impactors' lower 50% cutpoint thereby increased from about 0.15 to as much as 0.25 µm - still adequate to concentrate the majority of ambient fine (accumulation-mode) particles. If pressure drop again increased, the major flow from stage 2 was stopped and the hoses disconnected for 1 to 2 min. This tended to cause release of impacted particles, resulting in a brief concentration spike in the chamber, and a return to more normal operation once major flow was restored. On a few occasions, however, these interventions were ultimately unsuccessful, and exposures had to be terminated early (see main report).

Figure D-3 illustrates the exposure chamber, a body plethysmograph modified by adding an extended footwell to accommodate a small pedal-crank exercise device. The chamber was constructed of Formica-sheathed plywood with a clear plastic door and windows, sealed by closed-cell foam strips. The inlet pipe faces the subject approximately at chest height. The main sampling port, through which air is drawn into a MOUDI at 30 L/min, is just above the inlet. Additional ports to the left and right of the inlet, not visible in the illustration, supply air to a HDS sampler and filter cassettes for carbon and elemental analysis, each drawing 10 L/min. A pump behind the chamber exhausts air, typically at 120 L/min, through 5 small ports at regular intervals in a pipe extending across the chamber just above/behind the subject's head. Through a port in the ceiling directly

above the subject's head, a Data RAM nephelometer draws air at 2 L/min for continuous concentration monitoring. To maintain the nephelometer sampling circuit at the same pressure as the chamber, the nephelometer exhaust air is returned to the chamber through another port in the upper front wall. Prior to exposure studies, the chamber was leak-checked by sealing the inlet and sampling ports, reducing the interior air pressure by briefly operating the exhaust pump. Leaks were then traced by sound, and sealed with silicone-based adhesive. Particle distribution inside the chamber was tested by a staff member inside holding a Data RAM nephelometer, moving the inlet systematically to different positions within, above, below, and to the side of the breathing zone, in randomized order to avoid confounding temporal with spatial variation. No statistically significant vertical concentration gradients were found. In one experiment with the concentrator disconnected and ultrasonically nebulized saline aerosol introduced to the chamber at a mean concentration of 1769 $\mu g/m^3$ by Data RAM, a statistically significant horizontal gradient was found, with an average 13% excess concentration on the right (door) side compared to the rest of the chamber. On two occasions with the concentrator operating normally and average in-chamber concentrations near 350 $\mu g/m^3$, significant horizontal gradients were found, with less than 10% excess concentration on the left side compared to the rest of the chamber. Given that observed gradients were small in magnitude, and inconsistent in direction in different experiments, the distribution of fine particles in the chamber was considered to be uniform for practical purposes.

AIR SAMPLING AND ANALYSIS

In addition to in-chamber monitoring by MOUDI and HDS (described in the main report), comparable MOUDI and HDS samplers were operated concurrently to sample air in the concentrator upstream of stage 1, downstream of the size-selective inlet. These samples were considered to represent the fine-particle content of ambient air during exposure studies. Table D-1 summarizes results of ambient sampling.

Each MOUDI employed seven 47-mm Teflon filters (Teflo, Fisher Scientific, 2 µm pore size) in the upper stages, and one 37-mm Teflon filter as the backup. Each HDS employed an upstream sodium-carbonate-coated honeycomb denuder to remove acidic gases (which was not analyzed in this study), a 47-mm Teflon main filter, and a 47-mm sodium-carbonate-coated glass fiber backup filter to capture that portion of particulate nitrate volatilized from the main filter. The nitrate measured on the backup was assumed to be ammonium nitrate, and the appropriate equivalent mass was added to the mass measured on the main filter to obtain the HDS measurement of total mass concentration. All filters were pre- and post-weighed to determine mass concentrations, as described in the main report. Temperature/humidity equilibration for 24 hr preceded each weighing. The weighing room was maintained between 21 and 27 degrees C, 40% and 60% relative humidity. The limit of detection (LOD) for the balance, defined as 3 times the standard deviation of repeated weighings of a blank Teflon filter, was <= 8 µg , yielding a concentration LOD near 2 $\mu g/m^3$ for any stage of the MOUDI and 7 $\mu g/m^3$ for the HDS. After weighing, filters were wetted with 200 µl ethanol, then extracted with 10 mL of ultra-pure water and sonicated 30 min. Sulfate and nitrate content of the extracts was determined by ion chromatography, as described in the main report. A three-point calibration of the ion chromatograph was performed immediately before analyzing each batch of filters. (A batch typically included all filters from two exposure studies.) Five percent of samples were analyzed twice to verify reproducibility (relative mean deviation of the two measurements < 10%). The LOD, redetermined every 6 months, were defined as 3 times the standard deviation of 7 to 9 repeated measurements of a prepared standard, with concentration approximately 5 times the expected LOD. For nitrate, the highest observed LOD was 0.7 µg, equivalent to a concentration of 1.6 $\mu g/m^3$ for the MOUDI (all stages) or 1.2 $\mu g/m^3$ for the HDS. For sulfate, the highest observed LOD was 0.9 µg, equivalent to a concentration of 2 $\mu g/m^3$ for the MOUDI or 1.5 $\mu g/m^3$ for the HDS. Nitrate and sulfate values below the LOD were recorded as zero.

Tissue quartz filters for carbon sampling (Pall Gelman Sciences, 47 mm) were preheated and coated at the analytical laboratory (AtmAA Inc., Calabasas CA) before shipment to our laboratory, where they covered with aluminum foil, sealed in plastic bags, and stored in a vacuum desiccator until use. After exposure, they were stored in Petri dishes sealed with Teflon tape, covered with aluminum foil, placed in sealed plastic bags inside larger sealed plastic bags, and refrigerated before and during shipment back to the analytical laboratory.

Filters for elemental analysis (Teflon 47 mm) were stored and weighed before and after exposure in the climate-controlled weighing room, like the similar MOUDI filters. They were shipped to Chester LabNet, Tigard, OR, for analysis by X-ray fluorescence. Table D-2 presents detailed elemental analysis results not included in the main report.

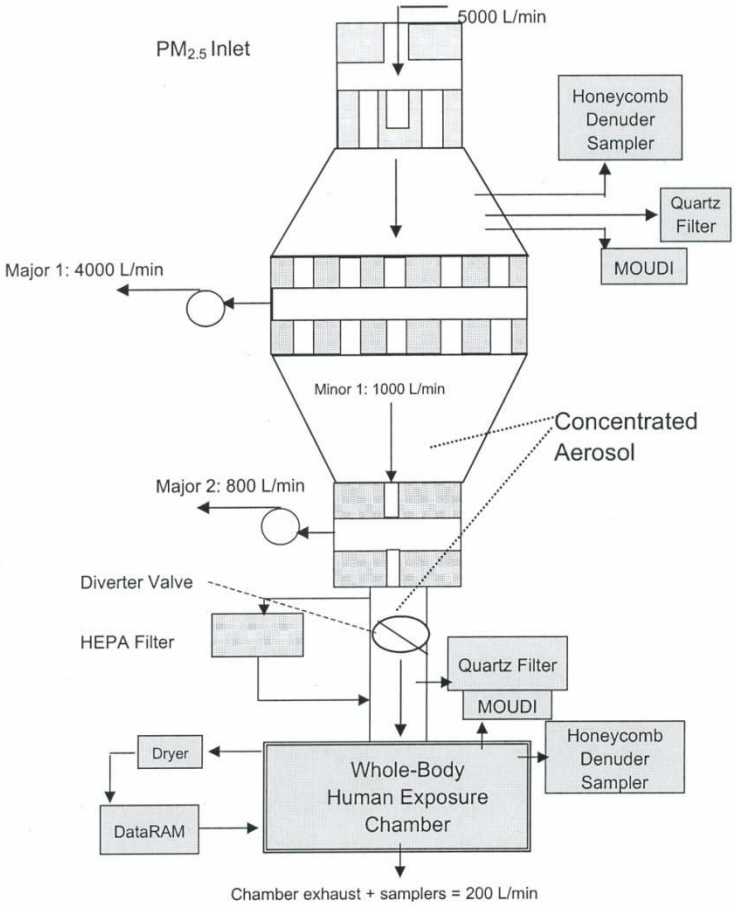


Figure D-1. Schematic diagram of ambient fine particle concentrator, exposure chamber, and monitoring instruments (filter sampling for elemental analysis not shown).

Figure D-2. Concentrator stage 1, viewed from upstream side with access hatch removed to show 5 impactor slits in parallel. Major flow exhaust hoses are visible to left of impactor slits.

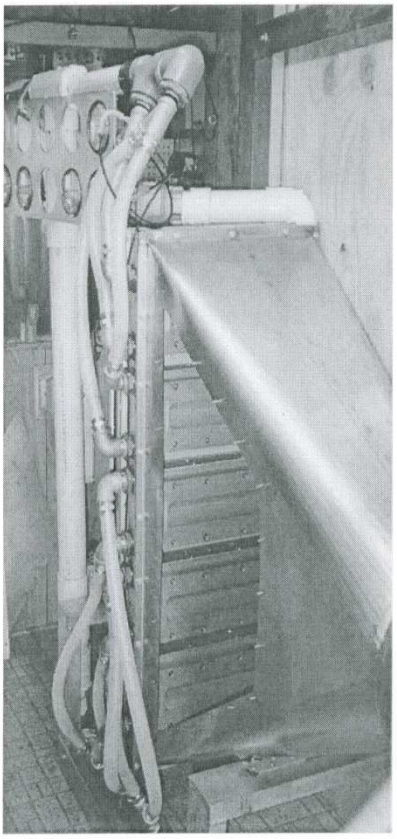
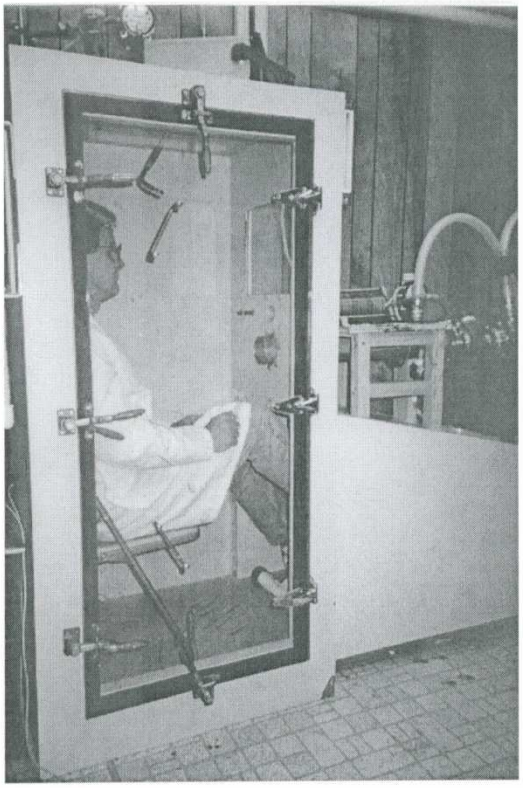


Figure D-3. Side view of exposure chamber with staff member inside in subject's usual position. (Actual subjects wore clean-room clothing when inside.) Inlet pipe (larger opening) and main (MOUDI) sampling port (smaller opening) are visible in front wall below window. Sampling instrument platform and hoses of HEPA-filter-containing bypass circuit are visible outside upper front of chamber.



Appendix Available on Request

TABLE D-1. AMBIENT FINE PARTICLE CONCENTRATION MEASUREMENTS
(MICROGRAMS/CUBIC METER) DURING CONCENTRATED PARTICLE EXPOSURES

GROUP	ID #	< MOUDI >			< HDS >		
		MASS	NO3	SO4	MASS	NO3	SO4
Healthy	1082	93.1	4.8	5.0	42.2	4.4	6.1
Healthy	2172	71.9	18.8	2.2	87.4	20.4	1.7
Healthy	2324	74.4	12.6	4.3	81.0	15.6	5.3
Healthy	2325	41.0	5.2	3.9	54.4	5.0	2.6
Healthy	2340	49.4	5.8	2.7	96.0	7.2	4.4
Healthy	2459	85.6	24.5	9.2	79.5	25.4	9.7
Healthy	2461	63.9	10.4	5.8	84.6	13.6	5.7
Healthy	2465	74.4	14.2	3.8	103.1	15.7	3.2
Healthy	2476	82.5	21.6	2.2	103.4	23.8	2.7
Healthy	2503	65.8	9.4	1.0	70.0	11.8	1.2
Healthy	2505	34.2	4.0	0.1	36.5	6.1	0.0
Healthy	2508	115.2	32.4	2.8	167.3	34.8	3.0
Asthma	1656	51.4	7.5	1.1	22.5	10.9	0.0
Asthma	1750	60.4	3.0	0.0	53.3	3.8	0.0
Asthma	1808	64.7	12.1	3.5	55.3	11.7	3.3
Asthma	2216	59.2	10.8	6.8	86.2	15.6	6.2
Asthma	2433	44.4	4.0	4.0	68.3	2.6	4.7
Asthma	2525	35.0	5.3	4.7	70.2	8.7	5.3
Asthma	2528	62.5	6.1	2.9	52.6	8.3	4.4
Asthma	2541	75.9	0.0	0.0	4.2	3.2	0.0
Asthma	2543	32.5	5.6	0.3	37.3	8.3	0.0
Asthma	2550	69.6	0.7	4.0	53.1	4.1	3.7
Asthma	2551	49.4	11.7	1.1	44.5	14.2	1.4
Asthma	2563	43.3	0.9	9.6	43.4	2.6	8.3

Appendix Available on Request

Table D-2. Concentrated Ambient Particle Mass Concentrations from Total Filter, and Element Concentrations from X-Ray Fluorescence Analysis (Chronological Order)

YR	MON	DY	ASTH	SUB	MASS	Al	Si	P	S	Cl	K	Ca	Ti	V	Cr	Mn	Fe	Co	Ni	Cu
1999	7	13	0	1082	266.67	6.28	17.63	0.02	6.98	3.78	3.32	10.61	0.99	0.00	0.05	0.13	7.22	0.00	0.05	0.18
1999	7	22	0	2325																
1999	8	17	0	2340	169.17	0.96	2.61	0.00	6.20	1.15	0.84	2.30	0.42	0.02	0.01	0.05	2.15	0.00	0.02	0.07
1999	8	31	0	2461	155.00	1.92	2.80	0.00	6.12	0.58	0.52	1.71	0.17	0.00	0.01	0.04	1.22	0.01	0.01	0.04
1999	9	21	0	2324																
1999	9	28	0	2459	155.00	0.40	1.61	0.00	6.68	0.83	0.44	1.05	0.34	0.02	0.01	0.03	0.97	0.01	0.01	0.12
1999	11	10	0	2476	226.67	0.73	2.32	0.00	3.39	3.90	0.99	1.89	0.17	0.00	0.01	0.03	1.49	0.00	0.02	0.07
1999	12	14	0	2172	270.00	3.55	8.86	0.00	4.10	5.97	2.34	4.39	0.59	0.02	0.04	0.18	4.45	0.00	0.03	0.22
1999	12	20	0	2503	266.67	6.70	13.16	0.13	2.81	8.51	3.88	7.30	0.64	0.00	0.02	0.21	8.77	0.00	0.02	0.17
2000	1	4	0	2505	154.17	1.71	5.03	0.00	1.51	3.02	0.97	2.88	0.37	0.00	0.02	0.17	5.53	0.00	0.02	0.13
2000	1	13	0	2508	298.89	4.30	11.58	0.08	4.57	6.31	2.41	5.57	0.54	0.00	0.01	0.12	5.19	0.00	0.03	0.37
2000	2	8	0	2465	258.33	2.33	6.45	0.00	6.74	4.44	1.69	4.63	0.66	0.07	0.02	0.13	4.39	0.00	0.02	0.13
2000	4	13	1	2216	194.17	1.31	3.57	0.00	7.97	1.13	1.88	1.97	0.28	0.06	0.03	0.07	1.93	0.01	0.05	0.14
2000	5	31	1	2433	228.33	0.00	1.74	0.02	10.74	1.32	0.70	0.88	0.12	0.03	0.02	0.03	0.98	0.00	0.00	0.09
2000	6	6	1	2528	236.67	0.69	3.62	0.00	7.98	1.53	1.10	2.27	0.31	0.06	0.02	0.08	2.09	0.00	0.02	0.09
2000	8	17	1	2525	219.17	2.06	3.63	0.00	10.70	1.51	1.92	3.57	0.41	0.08	0.02	0.08	2.80	0.00	0.02	0.21
2000	11	7	1	2541	122.22	4.63	8.27	0.02	2.16	0.72	1.34	4.24	0.16	0.00	0.03	0.12	5.09	0.00	0.01	0.12
2000	11	14	1	1656	230.83	1.86	3.27	0.07	4.50	3.30	1.40	3.45	0.43	0.06	0.02	0.10	3.77	0.00	0.02	0.17
2000	12	28	1	2543	198.33	3.17	5.10	0.02	2.56	10.40	1.76	3.78	0.39	0.02	0.03	0.12	4.92	0.02	0.01	0.18
2001	1	17	1	1750	173.33	1.98	4.23	0.04	3.14	1.57	1.43	2.86	0.69	0.06	0.09	0.33	7.79	0.02	0.09	0.21
2001	2	15	1	2551	221.67	1.24	2.24	0.01	3.74	1.51	0.98	1.61	0.27	0.03	0.02	0.12	3.13	0.01	0.03	0.14
2001	2	22	1	2550	234.44	0.29	3.42	0.03	14.14	2.51	0.91	1.84	0.41	0.03	0.06	0.06	1.93	0.01	0.03	0.12
2001	3	27	1	1808	240.83	0.52	2.37	0.01	7.66	1.94	0.60	2.03	0.22	0.03	0.02	0.03	1.62	0.01	0.01	0.08
2001	7	10	1	2563	172.50	0.59	2.96	0.00	22.43	0.09	0.77	1.77	0.42	0.11	0.04	0.06	1.64	0.00	0.02	0.06
				Mean	213.32	2.15	5.29	0.02	6.67	3.00	1.46	3.30	0.41	0.03	0.03	0.10	3.59	0.00	0.03	0.14
				SD	46.40	1.89	4.17	0.03	4.72	2.69	0.89	2.26	0.21	0.03	0.02	0.07	2.30	0.01	0.02	0.07

Appendix Available on Request

(Table D-2 continued)

YR	MON	DY	ASTH	SUB	MASS	Zn	As	Se	Br	Rb	Sr	Y	Zr	Mo	Pb
1999	7	13	0	1082	266.67	1.92	0.00	0.01	0.04	0.01	0.08	0.00	0.19	0.00	0.15
1999	7	22	0	2325											
1999	8	17	0	2340	169.17	0.42	0.02	0.03	0.04	0.00	0.01	0.00	0.00	0.04	0.10
1999	8	31	0	2461	155.00	0.14	0.00	0.00	0.02	0.00	0.02	0.00	0.03	0.00	0.06
1999	9	21	0	2324											
1999	9	28	0	2459	155.00	0.20	0.01	0.00	0.03	0.00	0.00	0.00	0.02	0.00	0.10
1999	11	10	0	2476	226.67	0.47	0.02	0.03	0.04	0.01	0.01	0.00	0.02	0.00	0.10
1999	12	14	0	2172	270.00	0.91	0.03	0.02	0.08	0.00	0.05	0.00	0.26	0.00	0.18
1999	12	20	0	2503	266.67	0.94	0.02	0.02	0.05	0.01	0.08	0.00	0.19	0.02	0.22
2000	1	4	0	2505	154.17	1.03	0.02	0.00	0.03	0.00	0.03	0.00	0.20	0.02	0.17
2000	1	13	0	2508	298.89	1.08	0.00	0.00	0.09	0.00	0.07	0.00	0.13	0.00	0.11
2000	2	8	0	2465	258.33	0.76	0.01	0.00	0.07	0.01	0.07	0.00	0.26	0.00	0.11
2000	4	13	1	2216	194.17	0.41	0.01	0.01	0.02	0.00	0.02	0.00	0.47	0.00	0.06
2000	5	31	1	2433	228.33	0.13	0.00	0.01	0.06	0.01	0.02	0.00	0.11	0.03	0.08
2000	6	6	1	2528	236.67	0.47	0.02	0.01	0.03	0.02	0.04	0.01	0.30	0.00	0.03
2000	8	17	1	2525	219.17	0.44	0.02	0.02	0.07	0.00	0.08	0.00	0.16	0.00	0.12
2000	11	7	1	2541	122.22	0.54	0.04	0.03	0.00	0.01	0.02	0.00	0.03	0.00	0.10
2000	11	14	1	1656	230.83	0.61	0.01	0.01	0.12	0.00	0.03	0.00	0.21	0.00	0.14
2000	12	28	1	2543	198.33	1.01	0.02	0.00	0.03	0.00	0.04	0.00	0.06	0.06	0.17
2001	1	17	1	1750	173.33	0.94	0.04	0.00	0.03	0.00	0.06	0.00	0.08	0.01	0.10
2001	2	15	1	2551	221.67	0.40	0.00	0.02	0.06	0.01	0.03	0.00	0.13	0.09	0.12
2001	2	22	1	2550	234.44	0.52	0.00	0.02	0.07	0.01	0.03	0.00	0.02	0.00	0.06
2001	3	27	1	1808	240.83	0.27	0.00	0.00	0.06	0.02	0.02	0.00	0.08	0.00	0.07
2001	7	10	1	2563	172.50	0.19	0.00	0.01	0.04	0.00	0.03	0.00	0.02	0.00	0.03
				Mean	213.32	0.63	0.01	0.01	0.05	0.00	0.04	0.00	0.14	0.01	0.11
				SD	46.40	0.42	0.01	0.01	0.03	0.01	0.02	0.00	0.12	0.02	0.05

Appendix 2

Blood urine stats pre-FA all subjects, variable names "l.." are log transformed

The MEANS Procedure

Variable	Label	N	Mean	Std Dev	Lower Quartile	Upper Quartile	Minimum	Maximum	Skewness
serIgE	serIgE	30	6.3297667	3.5607293	2.851	9.021	0.496	13.419	-0.1205392
serIgA	serIgA	30	1445.53	1780.97	530.967	1836.42	156.494	9718.01	3.6668711
serIgG	serIgG	29	565.5145862	892.144171	63.441	603.92	36.407	4495.8	3.3785247
serIgM	serIgM	27	2906.79	6975.62	142.944	2722.93	96.768	32705.33	3.6161771
serIgG4	serIgG4	28	463.9671071	1295.51	44.9325	233.0865	19.101	5964.94	3.7543832
serIL8	serIL8	30	125.4635667	254.8152899	12.055	100.12	1.154	1175.09	3.2534359
CRP	CRP	30	29.3074	39.2958161	4.407	37.756	0.26	181.763	2.4160642
Fib	Fib	30	929.44866	609.3036464	373.646	1224.56	58.728	2615.01	0.7914139
Fact7	Fact7	30	19.557	9.5392426	13.36	22.273	8.652	53.572	1.8430082
vWF	vWF	30	61.7357	24.9823871	45.561	85.803	25.299	119.903	0.589719
Ur_Isopros	Ur_Isopros	27	0.0849815	0.0994218	0.013	0.129	0.0005	0.331	1.2270519
lserige		30	0.6905697	0.3692872	0.4549972	0.9552547	-0.3045183	1.1277202	-1.1691298
lseriga		30	2.9529229	0.4368964	2.7250675	3.2639725	2.1944977	3.9875772	-0.0555858
lserigg		29	2.3828826	0.5835836	1.80237	2.7809794	1.5611849	3.652807	0.2592101
lserigm		27	2.7406079	0.7436647	2.1551659	3.4350365	1.9857318	4.5146185	0.9878513
lserigg4		28	2.0297265	0.5991923	1.6506142	2.3626701	1.2810561	3.7756062	1.5476983
lseril8		30	1.5695256	0.6705588	1.0811672	2.0005208	0.0622058	3.0700711	0.3469255
lcrp		30	1.0616815	0.6903172	0.6441431	1.576986	-0.5850267	2.2595055	-0.400147
lfib		30	2.8399494	0.3939488	2.5724603	3.0879811	1.7688452	3.4174734	-1.0944156
lfact7		30	1.251635	0.1807556	1.1258065	1.3477787	0.9371165	1.7289379	0.7279874
lvwf		30	1.7553956	0.180463	1.6585932	1.9335025	1.4031034	2.07883	-0.168068
luriso		27	-1.4732616	0.7166428	-1.8860566	-0.8894103	-3.30103	-0.480172	-0.6167123

Variable	Label	Kurtosis
serIgE	serIgE	-1.0482901
serIgA	serIgA	16.5496892
serIgG	serIgG	13.6629696
serIgM	serIgM	13.8326708
serIgG4	serIgG4	13.9007503
serIL8	serIL8	10.9909188
CRP	CRP	7.1446126
Fib	Fib	0.8578559
Fact7	Fact7	4.3633664
vWF	vWF	-0.4824321
Ur_Isopros	Ur_Isopros	0.2359494
lserige		0.5536521
lseriga		-0.1758252
lserigg		-0.947765
lserigm		-0.1600809
lserigg4		2.5536307
lseril8		0.214342
lcrp		-0.4278026
lfib		0.7741058
lfact7		0.169896
lvwf		-0.5719162
luriso		0.0896729

Blood urine stats change post-pre FA all subjects, untransformed

The MEANS Procedure

Variable	Label	N	Mean	Std Dev	Lower Quartile	Upper Quartile	Minimum	Maximum	Skewness
IgEchg	IgEchg	30	-0.15	0.834879	-0.468	0.212	-2.398	1.877	-0.4084813
IgAchg	IgAchg	30	-180.0972333	-484.7316681	404.501	-10.403	-1251.98	9 54.481	0.2531566
IgGchg	IgGchg	30	12.6851	-1412.26	268.919	-3.057	-2832.24	6685.06	3.5454397
IgMchg	IgMchg	28	-491.0901429	4879.67	-73.615	185.469	-16743.52	13468.39	-1.1670923
IgG4chg	IgG4chg	28	-175.6940714	745.2334196	-4.517	8.0485	-3838.2	27.282	-4.787069
IL8chg	IL8chg	30	20.7660333	112.352042	-1.795	4.995	-188.6600000	5 22.055	3.3852739
CRPchg	CRPchg	30	7.5255	17.9588391	-0.995	6.767	-10.913	88.156	3.4405829
Fibchg	Fibchg	30	29.90534	564.3837652	-73.079	319.274	-2041.46	9 11.025	-2.0640072
Fact7chg	Fact7chg	30	1.5074667	5.5673888	-0.183	3.614	-17.146	16.019	-0.7025199
vWFchg	vWFchg	30	1.8620333	19.5627568	-6.847	10.618	-49.777	44.404	-0.3502207
Isopchg	Isopchg	26	0.0638269	0.3290006	-0.028	0.034	-0.23	1.614	4.4785965

Variable	Label	Kurtosis
IgEchg	IgEchg	1.8724148
IgAchg	IgAchg	0.9169725
IgGchg	IgGchg	18.577398
IgMchg	IgMchg	7.3859248
IgG4chg	IgG4chg	23.7214915

IL8chg	IL8chg	14.8553029
CRPchg	CRPchg	14.2680005
Fibchg	Fibchg	6.4957718
Fact7chg	Fact7chg	4.5976354
vWFchg	vWFchg	1.0171348
Isopchg	Isopchg	21.7304646

Blood urine stats change in log-transformed data post-pre FA all subjects

The MEANS Procedure

Variable	Label	N	Mean	Std Dev	Lower Quartile	Upper Quartile	Minimum	Maximum	Skewness
LIgEchg	LIgEchg 3	0	-0.0090153	0.0487021	-0.0402523	0.0148656	-0.108442	0.103988	0.0403639
LIgAchg	LIgAchg 3	0	-0.1140176	0.1449867	-0.2196282	-0.0136349	-0.3967456	0.1424249	-0.1991285
LIgGchg	LIgGchg 3	0	0.0018968	0.6060974	-0.3181195	-0.0164883	-0.7195766	2.6209852	3.0003651
LIgMchg	LIgMchg 2	8	0.1302564	0.8129997	-0.0787197	0.064672	-0.5488209	4.1293155	4.6942973
LIgG4chg	LIgG4chg 2	8	-0.0108256	0.1090225	-0.0530055	0.0398308	-0.4478915	0.1650926	-2.3865832
LIL8chg	LIL8chg 3	0	0.0240858	0.1657754	-0.045281	0.1033353	-0.6134054	0.355121	-1.5567912
LCRPchg	LCRPchg 3	0	0.0085161	0.4405041	-0.1318143	0.1919287	-1.7198638	0.8912813	-2.066153
LFibchg	LFibchg 3	0	0.1060836	0.3279161	-0.0416407	0.1609434	-0.6588998	0.849788	0.6724339
LFact7chg	LFact7chg 3	0	0.0278604	0.1166754	-0.0060226	0.085464	-0.3839768	0.2590453	-1.4187371
LvWFchg	LvWFchg 3	0	0.0027155	0.1424296	-0.0603311	0.0654511	-0.382397	0.2534087	-0.8715588
LIsopchg	LIsopchg 2	6	0.0739763	0.6213482	-0.2668159	0.3802112	-1.69897	1.2552725	-0.5346792

Variable	Label	Kurtosis
LIgEchg	LIgEchg	0.3110354
LIgAchg	LIgAchg	-0.6059705
LIgGchg	LIgGchg	11.8468961
LIgMchg	LIgMchg	23.7664902
LIgG4chg	LIgG4chg	9.2055929
LIL8chg	LIL8chg	7.125112
LCRPchg	LCRPchg	8.0066312
LFibchg	LFibchg	1.4562647
LFact7chg	LFact7chg	4.7779723
LvWFchg	LvWFchg	1.1343918
LIsopchg	LIsopchg	1.5384666